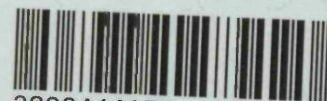


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University of London

Doctor of Medicine (MD) Thesis

2007

**Expression of brain derived neurotrophic
factor (BDNF), neurotrophin 4 (NT4) and
their common receptor, TrkB, by human
Müller cells
in vitro and *in vivo***

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Abstract

Neurotrophins are trophic and mitogenic proteins that play a role in the development, differentiation, connectivity and survival of neurons, acting via their specific receptors in the central and peripheral nervous system, including the retina. The preferred receptor for brain derived neurotrophic factor (BDNF) and neurotrophin 4 (NT4) is TrkB. Müller cells may play an important role in neurotrophin function in the nervous system. It has been suggested that neurotrophins exert their effects on photoreceptors by acting indirectly through activation of Müller cells.

The aims of the present work were to characterise the expression of BDNF, NT4 and TrkB by human Müller cells in vitro, and to examine whether changes in the expression of these molecules occur in retina from patients with proliferative vitreoretinopathy (PVR) when compared with normal retina. In addition, changes in the expression of BDNF, NT4 and glial fibrillary acidic protein (GFAP) were also investigated in melanoma affected human retina following laser photocoagulation.

A variety of techniques were employed to investigate the expression of the above neurotrophins and TrkB at the RNA and protein levels. These included cell culture and mRNA extraction,

RT-PCR, Western blot, and immunocytochemistry. Confocal laser scanning microscopy and light microscopy were used for imaging.

The results showed that cultured human Müller cells express BDNF and NT4 as indicated by both mRNA and protein expression. A truncated isoform of TrkB was also shown to be expressed by a spontaneously immortalized human Müller cell line used in the study (MIO-M1).

Staining for NT4 was greatly increased in retinal sections from eyes with PVR, compared with normal retina. NT4 expression by Müller cells *in situ* was confirmed by confocal imaging observations that cells staining for this neurotrophin co-stained for GFAP. By contrast, there was a decrease in TrkB immunostaining in PVR retinectomy sections compared with controls. NT4 staining was also reduced at the site of laser burns in melanoma affected retina. Possible explanations are discussed in the thesis.

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List of abbreviations

α-SMA	α-smooth muscle actin
ARMD	age related macular degeneration
BDNF	brain derived neurotrophic factor
bFGF	basic fibroblastic growth factor
CMO	cystoid macular oedema
CNTF	ciliary neurotrophic factor
CRALBP	cellular retinaldehyde binding protein
CREB	cAMP responsive element binding protein
DMEM	Dulbecco's modified Eagle's medium
EGF-R	epidermal growth factor receptor
ERG	electroretinogram
ERM	epiretinal membrane
GCL	ganglion cell layer
GFAP	glial fibrillary acidic protein

INL	inner nuclear layer
IPL	inner plexiform layer
MIO-M1	Moorfields, Institute of ophthalmology- Müller 1
MAPK	mitogen activated protein kinase
NT	neurotrophins
NT3	neurotrophon 3
NT4	neurotrophin 4
NFL	nerve fibre layer
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
ONL	outer nuclear layer
OPL	outer plexiform layer
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PVR	proliferative vitreoretinopathy
PR	photoreceptor

RD	retinal detachment
RPE	retinal pigment epithelium
RT-PCR	reverse transcription-polymerase chain reaction
TGF-β	transforming growth factor β
TNF	tumor necrosis factor
TrkA	tropomyosin-related kinase A
TrkB	tropomyosin-related kinase B
TrkC	tropomyosin-related kinase C
Trk-FL	tropomyosin-related kinase-full length
t-Trk	truncated tropomyosin related kinase
VEGF	Vascular endothelium growth factor

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Chapter 1

Introduction

1.1 Retinal cells and structure

The retina (Latin: rete = net) is the internal layer of the eyeball. It is a thin delicate layer of nervous tissue that varies in thickness in different regions from about 0.1 mm at the most peripheral area (ora serrata) to 0.56 mm near the optic disc. Its thickness at the equator is 0.18mm (Sigelman J and Ozanics V1982; Tripathi RC and Tripathi BJ 1984; Ogden T 1989a and b). The surface area of the retina is approximately 266 mm². The central area of the retina (area centralis) is divided into the fovea and the foveola. The fovea is approximately at the centre of the area centralis and has a thickness of 0.25 mm and a diameter of 1.85 mm. It is located 4 mm temporal to the optic disc and about 0.8 mm below the horizontal meridian. The Foveola is located at the central part of the fovea and recognised by the concave indentation produced by its thinner structure. It measures 0.13 mm in thickness and 0.35 mm in diameter and is composed solely of cone photoreceptors. The macula lutea is about 3mm in diameter and is an oval, yellowish area, in the central retina. The yellow appearance is probably due to the presence of xanthophylls (a carotinoid pigment) in the ganglion and bipolar cells.

The retina has a highly organised structure and histologically has two main components: a pigmented layer and a sensory layer derived from the outer and the inner layers of the embryonic optic vesicle respectively. As seen in cross-section histologically, the retina is composed of 10 layers which from the outer to the inner aspect are: retinal pigment epithelium (RPE), photoreceptor layer of rods and cones, external limiting membrane,

outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fibre layer and inner limiting membrane.

At the fovea only the following layers are present: RPE, cone photoreceptors, the external limiting membrane, the outer nuclear layer, the inner fibres of the photoreceptors (Henle's fibre layer) and the internal limiting membrane.

The retinal pigment epithelium (RPE) has many important roles in the normal visual process including maintaining adhesion of the neurosensory retina, phagocytosis of the photoreceptor outer segments, improving image resolution by light absorption, transport and storage of metabolites and providing a permeable barrier between the choroid and the neurosensory retina.

The neurosensory retina consists of several cell types that form three layers of nerve cell bodies and two layers of synapses. Retinal cell types are divided between a population of non-neuronal cells including vascular endothelial cells, pericytes, and glial cells and neuronal cells including photoreceptors, horizontal and amacrine cells, bipolar cells and ganglion cells.

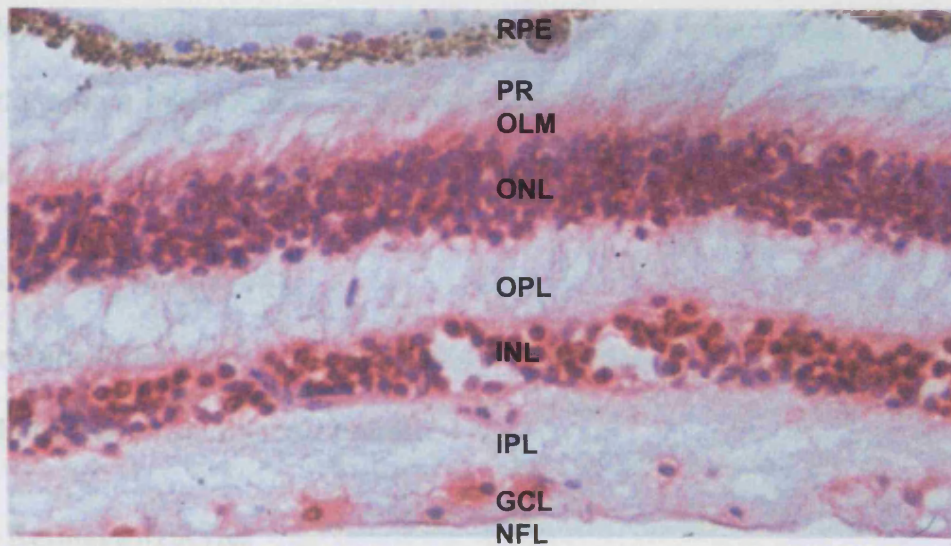


Figure 1.1 Layers of normal human retina (from Ghazi-Nouri SM *et al* 2003).

RPE=retinal pigment epithelium, PR=photoreceptor layer of rods and cones, OLM=outer limiting membrane, ONL=outer nuclear layer, OPL=outer plexiform layer, INL=inner nuclear layer, IPL=inner plexiform layer, GCL=ganglion cell layer, NFL=nerve fibre layer.

Neurones are the predominant cells in the retina. The light impulses are relayed by the photoreceptors, bipolar cells and ganglion cells and are transmitted to the brain via the ganglion cell axons in the optic nerve.

The outer nuclear layer contains cell bodies of the rods and cones, the inner nuclear layer contains cell bodies of the bipolar, horizontal, Müller and amacrine cells, and the ganglion cell layer contains cell bodies of ganglion cells and displaced amacrine cells. The outer and inner

plexiform layers (OPL and IPL) are where the synaptic contacts occur. In the OPL connections between rod and cones, and vertically running bipolar cells and horizontally oriented horizontal cells occur. The IPL functions as a relay station for the vertical-information-carrying nerve cells, the bipolar cells, to connect to ganglion cells.

1.2 Glial cells of the retina

There are three basic types of glial cells in the retina; astroglia, microglia and Müller cells as outlined below.

1.2.1 Retinal astrocytes

Astrocytes, named for their stellate shape, are not glial cells of the retinal neuroepithelium but are believed to enter the developing retina from the brain along the developing optic nerve (Stone J and Dreher Z 1987, Chan-Ling T 1994). Their characteristic morphology includes a flattened cell body and a fibrous series of radiating processes. They express glial fibrillary acidic protein (GFAP) (Schnitzer J 1988) and their cell bodies are almost entirely restricted to the nerve fibre layer of the retina. The distribution of retinal astrocytes is determined by the retinal vasculature (Stone J and Dreher Z 1987). Astrocytes are more prominent on the optic nerve head and are absent in the avascular fovea or ora serrata. Astrocytes are arranged over the surface of the ganglion cell axon bundles as they enter the optic nerve head. The blood vessels running in and among the ganglion cell bundles are also covered by both processes and occasional cell body of an astrocyte. This arrangement suggests that

astrocytes are axonal and vascular glial sheaths and part of a blood-brain barrier. They contain glycogen and may provide glucose to the neurons.

1.2.2 Retinal microglial cells

Microglial cells are found in every layer of the retina and are of mesodermal origin (unlike Müller cells and astrocytes which are derived from the neuroectoderm). They have small cell bodies with a thin cytoplasmic rim around the nucleus and irregular short processes that often encircle retinal capillaries (Vrabec F 1970). The distribution of microglia is believed to be related to the pattern of cell death at different stages of retinal development (Hume DA 1983). In several mammals studied including in the monkey, the retinal microglia are usually seen in association with the retinal vasculature and are found in all retinal layers from the margin of the inner retina to the outer plexiform layer (Vrabec F 1970). In the rat however the distribution of the retinal microglia is somewhat different with microglial cells found throughout the thickness of the retina during the embryonic stage but only seen in the inner half of the retina in the differentiated stage (Ashwell KW 1989).

Retinal microglial cells are analogous to the histiocytes of the central nervous system, exhibiting similar phagocytic properties in response to injuries (Potts RA 1982, Ling EA 1982, Hume DA 1983, Ashwell KW 1989). In normal human retina, microglial cells are normally in a resting state but may become activated by nerve degeneration, inflammation and traumatic nerve lesions and can play an important role in

immunoregulation and tissue repair, neurodegeneration, autoimmune disease, vitreoretinopathy, diabetic retinopathy, glaucoma and age related macular degeneration (Chen L 2002).

1.2.3 Müller cells

Müller cell functions are summarised in table 1.1. Müller cells are specialised glial cells of the retina. They are found in all retinal regions, except optic nerve head, of all vertebrates studied. Müller cells were first described by Heinrich Müller (1820-1864), who was professor of topographical and comparative anatomy at Wurzburg, Germany, in an article entitled "On the Histology of the Retina", published on 15th May 1851 (Sarthy V and Ripps H 2001).

In the mammalian retina there are about 10^6 to 10^7 Müller cells (Robinson SR and Dreher Z 1990, Dreher Z 1992, Reichenbach A and Robinson SR 1995, Distler C and Dreher Z 1996) and their morphology varies depending on their retinal location. Those at the periphery tend to appear shorter with broader end feet and have lower density compared to cells located centrally (Uga S 1974, Rasmussen KE 1974, Dreher Z 1988, Gauer VP 1988, Reichenbach A 1989, Robinson SR and Dreher Z 1990).

Müller cells span the entire thickness of the retina from the inner limiting membrane to the outer limiting membrane, which consists of junctional processes of Müller cells and photoreceptors. Müller cells are in direct and close relation with all retinal cell types. Their nuclei are usually located in the middle of the inner nuclear layer (Dowling JE 1987).

Müller cells are responsible for the structural stabilisation of the retina and provide an orientation scaffold. They are responsible for maintaining the

layered arrangement of the retinal neurons and are also involved in ion homeostasis and glutamate recycling mechanism in the retina. There is a strong expression of K^+ channels on their plasma membrane, especially of inwardly rectifying K^+ (Kir) channels, that makes the plasma membrane of Müller cells highly permeable to K^+ (Brew H 1986, Nilius B 1988, Newman EA 1993, Ishii M 1997). This is an important role as removal of extracellular K^+ is crucial for normal retinal function. Active neurons release K^+ which is then redistributed by Müller cells into the subretinal space, blood vessels and the vitreous (Newman EA 1984, Karwoski CJ 1989, Reichenbach A 1992). Vitreous (and blood) is a main sink for the K^+ redistribution and it has been suggested that a certain minimal volume of vitreal fluid is essential for efficient buffering of intraretinal increases of potassium ions through the Müller cells. Replacing the vitreous with a medium that is not capable of dissolving K^+ (such as perfluorocarbon liquid or silicone oil) may cause long-lasting accumulation of potassium ion and consequent neurodegenerative and reactive gliosis occurs (Winter M 2000).

Müller cells exert their important role in survival of neurones not only by removal of metabolic waste, but also by providing trophic factors (Poitry S 2000). Müller cells produce lactate by metabolising glucose. Neurones take up lactate for their oxidative metabolism (Poitry-Yamate CL 1995). Photoreceptor apoptosis, retinal degeneration and proliferation of retinal pigment epithelium (RPE) can occur as a result of selective Müller cell death (Dubois-Dauphin M 2000).

Function	References
Glucose uptake and metabolism and release of metabolites for neuronal use	Magistretti PJ 1999, Mantych GJ 1993, Watanabe T 1994
Glutamate metabolism	Riepe RE and Norenberg MD 1997, Pow DV 1994
GABA metabolism	Hyde JC and Robinson N 1974
Acid-base regulation	Musser GL and Rosen S 1973
Retinoid metabolism	Bunt-Milam AH and Saari JC 1983, Saari JC 1978, 1982, Bok D 1984
K regulation	Newman EA 1984, Karwoski CJ 1989, Reichenbach A 1992
NO synthesis	Kobayashi M 2000, Roth S 1997, Goureau O 1999, Koeberle PD 1999
Neurotrophic factors release and supporting ganglion cell survival	Meyer-Frank A 1995, Raju TR and Bennett MR 1986, Garcia M 2002

Table 1.1 Functions of Müller cells in relation to their metabolic interactions with neurons.

Various growth factors are known to be produced by these cells including vascular endothelium growth factor (VEGF) (Jingjing L 1999, Eichler W 2000) which is a major factor implicated in retinal neovascularisation observed in disorders such as diabetic retinopathy, retinopathy of prematurity, choroidal neo-vascularisation, and following retinal vein occlusion.

Müller cells are also responsible for removal of extracellular glutamate, which is the major retinal neurotransmitter and is also toxic to neural cells at high concentration. They exert this function by producing the enzyme glutamine synthetase responsible for the conversion of glutamate into glutamine within Müller cells. Glutamate can be detected within Müller cells at an increased level when glutamine synthetase is experimentally inhibited (Pow DV and Robinson SR 1994). There is a reduction of this enzyme's activity in Müller cells of detached retina (Erickson PA 1987, Lewis GP 1999) leading to disruption of glutamate recycling and subsequent glutamate toxicity.

Müller cells can also express nitric oxide synthetase under pathological conditions (Kobayashi M 2000). During hypoxia and hypoglycaemia nitric oxide (NO) produced by Müller cells may increase the retinal blood flow (Roth S 1997). The NO synthesised by activated Müller cells can induce neuronal cell death although it is not known to be toxic to the Müller cells themselves (Goureau O 1999, Koeberle PD 1999).

It has been shown that release of neurotrophic factors from glial cells support the survival of ganglion cells in culture (Meyer-Franke A 1995). In rats Müller cell conditioned medium has been shown to support survival of

ganglion cells at birth but not at 6-day postnatal stage (Raju TR and Bennett MR 1986). In pigs however the protective role of Müller cell has been observed even in adult animals' cells in culture (Garcia M 2002).

1.3 MIO/M1 Cell line

Availability of a cell line for in vitro studies is a valuable tool for a better understanding of various aspects of cellular functions. *In vitro* investigations of Müller cell functions have been laborious due to tendency of these cells to differentiate rapidly in culture and therefore difficulty in obtaining pure cell population (Sarthy VP 1998). Previous Müller cell lines reported in the literature have been obtained from the rat retina (sarthy VP 1998, Roque RS 1997). Recently a human Müller cell line has been characterised (Limb GA 2002) and named MIO-M1, after the institutions where it was isolated (Moorfields, Institute of Ophthalmology-Müller 1). MIO-M1 cells are spontaneously immortalized cells expressing well known markers of Müller cells including epidermal growth factor receptor (EGF-R), glutamate synthetase, cellular retinaldehyde binding protein (CRALBP), α -smooth muscle actin (α -SMA), and vimentin but not glial fibrillary acid protein (GFAP). GFAP expression in mammalian Müller cells is at low level or may be absent (Bignami A 1979, Lewis GP 1994), and it inceases in culture (McGillemm GS 1998) and in pathological conditions such as injury (Lewis GP 1994, Hollander H 1991). Lack of GFAP expression by cultured MIO-M1 cells suggests these cells do not exhibit characteristics of activation.

In addition to expressing the above cell markers MIO-M1 cells have morphologic appearance under phase contrast and transmission electron microscopy consistent with those reported in the literature for glial Müller cells (Limb GA 2002).

Both astrocytes and Müller cells have the potential to express GFAP. Müller cells also express CRALBP but astrocytes do not (CRALBP is also expressed by RPE cells). It is therefore possible to differentiate between these two glial cells by employing both of these markers.

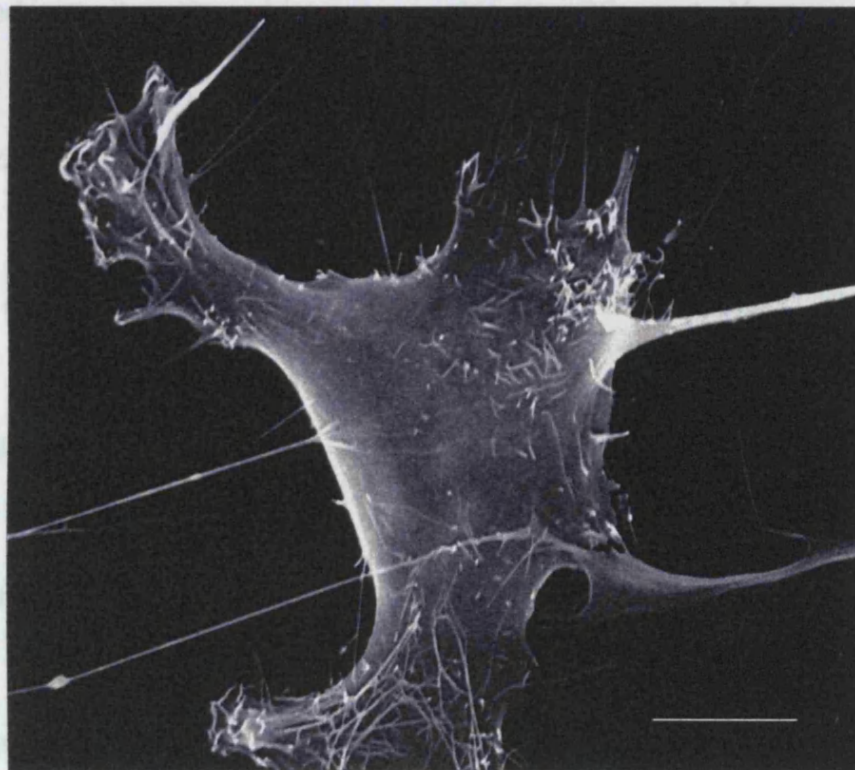


Figure 1.2 Scanning electron micrograph of a MIO-M1 cell (Courtesy of Dr Astrid Limb).

Scale bar=5 μ m

1.4 Retinal disorders associated with Müller cell dysfunction

Retinal disorders associated with Müller cell dysfunction are listed in the table 1.2. Dysfunction of Müller cells has been associated with a number of retinal disorders. These include X-linked retinoschisis, retinal gliosis in proliferative vitreoretinopathy (PVR) and epiretinal membrane (ERM), Müller cell sheen dystrophy, cystoid macular oedema, idiopathic macular hole formation and retinal neovascularisation.

Retinal Disorder	Reference
X-linked juvenile retinoschisis	Harris GS and Yeung J 1976, Condon GP 1986
Cystoid macular oedema	Fine BS 1981, Yanoff M1984, Loeffler KU 1992
Müller cell sheen dystrophy	Polk TD 1997, Kellner U 1998
Retinal detachment	Fisher SK 1994, Korte GE 1992
Retinal membranes/PVR	Guerin CJ 1990, Kono T 1995
Macular hole	Gass D 1999
Neovascularisation	Pierce EA 1995

Table 1.2 Retinal disorders associated with Müller cell dysfunction.

1.4.1 X-linked juvenile retinoschisis

This is an X-linked disease (affecting males and carried by females) first described by Haas in 1898 (Haas J 1898) and is a relatively rare vitreoretinal dystrophy. Its worldwide prevalence is between 1:15,000 to 1:30,000 (de la Chapelle A 1994) and causes bilateral splitting of the retina (retinoschisis) often involving the macula (foveomacular schisis). The mutation responsible for human X-linked retinoschisis has been identified and the gene codes for retinoschisin, which is expressed in photoreceptor and bipolar cells and is probably not related to Müller cells. Retinoschisin is a secreted protein containing a discoidin domain, which may be involved in cellular adhesion or cell-cell interactions (Tantri A 2004).

The exact pathogenesis of the condition is unknown however there are reports of an inherited defect and involvement of Müller cells in the pathogenic mechanism of the disease has been proposed (Yanoff M 1968, Manschot WA 1972, Condon GP 1986, Peachey NS 1987). Condon *et al* (Condon GP 1986) found intraretinal filaments produced by the defective Müller cells and proposed that their extracellular accumulation may lead to degeneration of cells and schisis formation. Retinoschisin has been shown to be selectively taken up and transported by Müller cells into the inner retina (Reid SN and Farber DB 2005). The electroretinogram (ERG) findings in affected cases typically show a reduced *b*-wave and normal *a*-wave. Miyake *et al* reported a reduction in the *a*-wave at the later stages of the disease (Miyake Y 1993). It is known that Müller cells are largely responsible for the *b*-wave component of an ERG and hence

Müller cell dysfunction has been suggested to be part of this abnormal retinal function. Sauer *et al* have identified a gene responsible for XLRS (called XLR1 gene) with several mutations being subsequently reported (Sauer CG 1997). Japanese juvenile retinoschisis is reported to be caused by mutations of the XLR1 gene (Hotta Y 1998).

Gene analysis in 234 familial and sporadic retinoschisis cases showed mutation in 214 cases with 82 different mutations. Despite the enormous mutation heterogeneity and regardless of the mutation type, patients had relatively uniform clinical manifestations. This and lack of symptoms in carriers suggests that retinoschisis is a disease caused by a loss-of-function rather than a dominant-negative effect (The Retinoschisis Consortium 1998).

Since Müller cells remove K^+ from the extracellular space, failure to do so will result in raised K^+ concentration and a transient white-yellow sheen might be seen in the retina (De Jong PT 1991). This phenomenon (known as Mizuo-Nakamura phenomenon) has been reported in X-linked retinoschisis, providing further support for involvement of Müller cells in the pathology of this condition. It has also been suggested that the colour changes associated with retinoschisis result from inability of the damaged cell end feet to clear K^+ from the extracellular space (De Jong PT 1991).

1.4.2 Cystoid Macular Oedema (CMO)

CMO is associated with a number of retinal conditions including retinovascular disease, retinal degeneration, intraocular inflammation, tumours, and post cataract surgery. It is thought that the condition is related to presence of clear cysts in Henle's layer and/or to the breakdown of blood retinal barrier with accumulation of serous exudates in the retina. It is unlikely that a single aetiological factor is responsible for the occurrence of CMO in such a wide variety of conditions. The question whether fluid is accumulated in extracellular space or within Müller cells is still not clearly understood. Some histopathological studies suggested that cystoid spaces represent swollen Müller cell processes (Fine BS 1981, Yanoff M 1984). Müller cell enlargement occurs without much accumulation of extracellular fluid suggesting that the primary site of oedema is the Müller cell itself (Fine BS 1981). In another more recent histopathologic study on the rare genetic condition dominantly inherited cystoid macular oedema primary disorder of the Müller cells was suggested (Loeffler KU 1992). Other studies suggest that fluid accumulation is in the extracellular space and that neuronal loss and Müller cell changes are secondary events (Gass JDM 1985, Tso MOM 1982, Wolter JR 1981).

1.4.3 Müller cell sheen dystrophy

This is an autosomal dominant condition causing multiple folds at the level of the internal limiting membrane (ILM) at the posterior pole of the retina. It can lead to macular oedema in some cases and the ERG changes show a reduction in the amplitude of the *b*-wave (Kellner U 1998). Histologically there is a diffuse thickening of the ILM, superficial retinal schisis cavities and cystoid spaces in the inner nuclear layer (Polk TD 1997). ILM thickening and folding has been suggested to be a result of production of a protein synthesized and secreted by Müller cells (Kellner U 1998). Schisis formation is also thought to be secondary to loss of structural support by Müller cells.

Currently there is no effective treatment available. Vitrectomy with membrane peel, systemic acetazolamide, non steroidal anti-inflammatory drugs (NSAID), steroids and grid laser photocoagulation all have been tried in the past but have failed to show a beneficial response (Polk TD 1997).

1.4.4 Retinal detachment and proliferative vitreoretinopathy

In rhegmatogenous retinal detachment (RD) there is a significant incidence of proliferative vitreoretinopathy (PVR) occurring in 5% to 10% of patients (Machemer R 1991, Asaria RH 2006). PVR is the commonest cause of failure of RD surgery and is characterised by cellular proliferation affecting both surfaces of the detached neuroretina as well as posterior vitreous face and within the vitreous base. These changes will result in

formation of contractile preretinal membranes. PVR can essentially be regarded as a wound healing process in a specialised neuronal tissue.

It is widely accepted that PVR involves a wide variety of cell types. These include epithelial cells from the RPE and the ciliary body, macrophages, lymphocytes, neutrophils, fibroblastic cells and glia cells including astrocytes and Müller cells. Subretinal membranes may take the form of a diffuse cell sheet or taut membranes or bands preventing reattachment of neuroretina. Diffuse cell sheets have been shown to be formed purely by glial cells (Wilkes SR 1987) whereas composition of subretinal bands is a mixture of RPE, fibroblasts like cells, macrophages and glia (Wilkes SR 1987, Trese MT 1985, Schwartz D 1988).

Involvement of glial cells in PVR has been demonstrated in several studies (Morino I 1990, Rodrigues MM 1981, van Horn DL 1977, Hiscott PS 1984). Glial cells proliferation can contribute to periretinal membrane formation. In animal models of RD, Müller cells undergo hypertrophy and proliferation within the retina and fill in the space left by dying photoreceptors (Lewis GP 2002). In the feline model Müller cells tend to grow into the subretinal space forming scars and preventing regeneration of photoreceptor outer segment after surgery leading to photoreceptor degeneration (Lewis GP 2002).

In PVR and subretinal fibrosis, RPE cells, Müller cells, and astrocytes proliferate continuously for weeks or months and migrate onto retinal surfaces. During PVR, cells attach to the ILM, proliferate, and form ERM. Contraction of this cellular membrane leads to recurrent RD. Müller cells have been identified in the ERM (Guerin CJ 1990) and in vitro they have

the ability to contract (Guidry C 1997, Mamballikalathil I 2000). It is therefore possible that Müller cells are involved in the contraction of these membranes.

1.4.5 Macular hole

Idiopathic or senile macular hole is a localised circular displacement of photoreceptors in the central retina. It affects approximately three in 1000 individuals resulting in variable reduction of central vision (La Cour M 2002).

It was proposed by Gass (Gass JDM 1999) that the structural support at the fovea is provided by a special cone-shaped arrangement of Müller cells (Müller cell cone), which serves as a plug to bind together the receptor cells in the foveola. Without this arrangement the thin photoreceptor cell layer would be susceptible to disruption and hole formation which may occur secondary to sensory retinal detachment, trauma, cystoid macular oedema, and macular degeneration.

It is also believed that in age-related macular hole formation the tractional forces caused by contraction of prefoveal vitreous cortex, which is firmly attached to the internal limiting membrane of the Müller cell cone, is responsible for the pathology (Gass JDM 1999,). Similar mechanism ie tangential tractional force caused by epiretinal membrane has been proposed in the pathogenesis of lamellar macular holes ERM tangential traction may play a role in the pathogenesis of lamellar macular holes (Guyon DR 1990).

1.4.6 Neovascularisation

Retinal neovascularisation is a major cause of blindness worldwide as it is associated with disorders such as retinopathy of prematurity, diabetic retinopathy, retinal vein occlusion, and subretinal neovascularisation in age related macular degeneration. It therefore can affect any age group from birth to working age population and the elderly and can lead to loss of vision. Hypoxia is a common factor in inducing retinal neovascularisation and Müller cells (as well as astrocytes and RPE cells) may have a crucial role in the pathogenesis of hypoxia-induced neovascularisation.

Vascular endothelium growth factor (VEGF) is a key regulator of normal and pathological retinal angiogenesis (Pierce EA 1995). The VEGF family is best known for its important roles in regulating the growth of vascular and lymphatic endothelia. There are a number of alternatively spliced forms of VEGF including variants of 121, 145, 165, 183, 189, and 206 (Dvorak HF 1999, Houck KA 1991, Tischer E 1991, Yang R 1996). The most abundant isoform of VEGF is 165 variant (Houck KA 1991). An important biological property differentiating between the VEGF isoforms is their heparin and heparan-sulfate binding ability. The 189 and 206 variants have enhanced heparin binding due to their structures containing stretches of basic residues. This will enable them to anchor to the extracellular matrix, while the 121 isoform is freely diffusible (Park JE 1993). The three secreted VEGF splice isoforms 121, 145 and 165 induce

proliferation of endothelial cells and *in vivo* angiogenesis (Poltorak Z 1997, Park J 1993, Zhang HT 1995). VEGF can be detected in both human plasma and serum samples. The detectable levels are higher in the serum due to its release from platelets (Banks RE 1998). VEGF expression is upregulated during hypoxia and is dependent upon the activities of the hypoxia-inducible transcription factors (HIF-1a and HIF-2a) (Maxwell P and Salnikow K 2004, Maxwell PH and Ratcliffe PJ 2002).

Low oxygen tension is an important stimulus for retinal VEGF synthesis and neovascularisation (Aiello LP 1994) and the release of VEGF by cultured Müller cells is increased during hypoxia (Aiello LP 1995, Jingjing L 1999, Eichler W 2000). Under normal conditions in the healthy retina, Müller cells release anti-angiogenic factors such as pigment epithelium derived growth factor (PEDF) and thrombospondin-1, which provide a permanent ant-proliferative condition for vascular endothelial cells (Eichler W 2004). PEDF is expressed in neurons and glial cells in the retina (Aymerich MS 2001, Ogata N 2002, Eichler W 2004) and acts as an anti-VEGF. Under conditions of low oxygen tension there is reduced PEDF expression in the retina and Müller cells (Duh EJ 2002, Eichler W 2004). A PEDF at low level is strongly associated with pregression of diabetic retinopathy (Boehm BO 2003). In conditions such as diabetic retinopathy and age related macular degeneration (ARMD), Müller cells have shown an increased expression of VEGF (Amin RH 1997). VEGF expression in Müller cells precedes neovascularisation in the diabetic patients without anatomical evidence of retinal malperfusion (Amin RH 1997). This

suggests that ischemia may not be the sole stimulus for VEGF expression in Müller cells (Amin HR 1997).

Furthermore, astrocytes and Müller cells express TGF-beta, which is a stimulant for VEGF expression by the Müller cells (Behzadian MA 1998). Treatment of cultured rat Müller cells with exogenous TGF-beta increases VEGF production both under normoxic and hypoxic conditions (Behzadian MA 1998). It is therefore suggested that TGF-beta activation may be prerequisite for hypoxia-induced up-regulation of VEGF and stimulation of angiogenesis in vivo (Behzadian MA 1998).

There is currently a growing interest in the use of Anti-VEGF agents in treatment of ARMD and diabetic retinopathy with encouraging reported successful outcomes.

Pegaptanib is a 28-nucleotide RNA aptamer specific for the VEGF 165 isoform and binds to it in the extracellular space, leaving other isoforms unaffected. This action inhibits such key VEGF actions as promotion of endothelial cell proliferation and survival, and vascular permeability (NG EW 2006). Intravitreal injection of Pegaptanib has been shown to be effective in treatment of choroidal neovascular membrane in age related macular degeneration (Gragoudas ES 2004) and in treatment of diabetic macular oedema (Cunningham ET Jr 2005) and its safety has been evaluated with favourable results in a two year follow up study (D'Amico DJ 2006).

Bevacizumab is another anti-VEGF antibody which was granted approval by the FDA in February 2004 for use as first-line therapy in metastatic colorectal cancer in combination with fluorouracil-based

chemotherapy. Bevacizumab binds to all biologically active isoforms of VEGF (Gordon MS 2001). It will therefore inhibit all biologic properties of VEGF including endothelial cell mitogenic activity, vascular permeability-enhancing activity, and those that promote angiogenesis (Fernando NH 2003). Both systemic and intravitreal administration of Bevacizumab has been shown to be effective in treatment of the neovascular age related macular degeneration (Rosenfeld PJ 2005, Michels S 2005).

Ranibizumab is derived from a humanized, monoclonal anti-vascular endothelial growth factor antibody and like bevacizumab neutralizes all active forms of vascular endothelial growth factor VEGF-A and its intravitreal injection has been shown to be effective in treatment of neovascular age related macular degeneration (Rosenfeld PJ 2006, Heier JS 2006).

Another way how Müller cells may be involved in hypoxia-induced pathologic angiogenesis is by release of transforming growth factor beta (TGF- β) and basic fibroblast growth factor (bFGF) (Frank RN 1996, Behzadian MA 1998). In hypoxia, Müller cells release the active form of the TGF- β whereas under normal oxygen tension the latent form is released (Behzadian MA 1998). Capillary endothelial cells are capable of releasing matrix metalloproteinase-9 (MMP-9) when in direct contact with Müller cells or astrocytes or when exposed to TGF- β (Behzadian MA 2001). Release of MMPs is associated with generation of leaky vessels as well as allowing the endothelial cells to penetrate their underlying basement membrane (Liotta LA 1980, Castilla MA 1999). It is believed that the TGF-

β induced release of MMP-9 is a potential cause of the blood-brain barrier breakdown at the onset of the angiogenesis (Behzadian MA 2001).

A further mechanism by which Muller cells may be involved in neovascularisation is via the rennin-angiotensin system. Renin is expressed in human retina and is specifically localized to the macroglial Müller cells. It has been suggested that retinal neovascularization may be associated with Müller cell dysfunction (Berka JL 1995).

1.5 Neurotrophins

Neurotrophins (NT) are trophic and mitogenic proteins that play a role in the development, differentiation, connectivity and survival of neurones in the central and peripheral nervous system, including the retina. NTs are of special interest to neurobiologists because they exert their biological action primarily on cells of the nervous system. These structurally related growth factors have unique activities on target neurones. They all have similar biochemical characteristics and are secreted as precursor proteins. These proneurotrophins were known to exist for quite some time, but their biological activity was only recently discovered (Lee R 2001). The biological action of neurotrophins is believed to be regulated by proteolytic cleavage, with precursor proteins preferentially activating p75 (see below section 1.6) to mediate apoptosis and mature forms activating Trk receptors (see below, section 1.6) to promote survival (Lee R 2001). The

mature part of all neurotrophins shows a high degree of sequence similarity.

Four NT have been identified in mammals: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4, which is also known as neurotrophin 5 (NT4/5). They are derived from a common ancestral gene and are similar in sequence and structure. NTs are expressed in regions that are invaded by sensory axons during development to provide them with trophic support en route to their final target (Farinas I 1996, 1998; Huang EJ 1999, Ringstedt T 1999). Many neurons have an autocrine role expressing BDNF (Mannion RJ 1999, Brady R 1999).

There are other growth factors, only briefly mentioned here, with neurotrophic effect. These factors are not part of the neurotrophins family because they are not derived from the same ancestral gene and include ciliary neurotrophic factors (CNTF) and basic fibroblastic growth factor (bFGF). CNTF has the biological effect on differentiation of photoreceptor cells and promoting the survival and axonal growth of ganglion cells (Whahlin KJ 2000, Chong NH 1999, Mey J 1993). In addition to affecting the differentiation of retinal neurons, CNTF also promotes Müller glia genesis in the postnatal mouse retina (Goureau O 2004).

NGF was the first NT described in 1950s by Rita Levi-Montalcini who was subsequently awarded the Noble prize in physiology and medicine in 1986 for this discovery. NGF was purified as a factor capable of supporting the survival of sympathetic and sensory spinal neurons in culture (Levi-Montalcini R 1987). It has been shown that NGF is synthesised and

secreted by cells of sympathetic and sensory target organs (Korsching S 1993 review). It is then captured by receptor-mediated endocytosis in nerve terminals and is transported through axons to neuronal cell bodies where it acts to promote neuronal survival and differentiation. There are other sources of NTs synthesis. Following peripheral nerve injury, macrophages infiltrate the nerve and release cytokines, which induce the synthesis of NGF in Schwann cells and fibroblasts within the injured nerve (Korsching S 1993 review). NGF synthesised in the damaged nerve is believed to be essential for survival and regeneration of injured neurons.

BDNF in its mature form is 52% identical to NGF at the amino acid level (Leibrock J 1989). Cells known to express BDNF include fibroblasts (Cartwright M 1994), astrocytes (Moretto G 1994), neurons (Barakat-Walter I 1996), megakaryocytes/platelets (Yamamoto H 1990) and retinal Müller cells (Oku H 2002). During development, BDNF has been implicated in neuronal differentiation, maturation, survival and synapse formation (Henderson CE 1996) whereas in the adult one of its most important roles is neuroprotection after injury (Kishino A 1997, Mansour-Robaey S 1994).

BDNF mRNA levels are known to be up regulated in response to ganglion cell axonal injury in rats. This observation suggests that endogenous BDNF may contribute to a natural neuroprotective process after optic nerve injury. To achieve this, target cells in the retina need to be expressing the appropriate receptor in the right isoform and at adequate levels (see section 1.6). BDNF may aid recovery of the detached retina

after reattachment (Lewis GP 1999) and ganglion cell regeneration following optic nerve injury (Mansour-Robaey S 1994).

The third member of the neurotrophin family to be discovered was NT3 (Ernfors P 1990). NT3 promotes the differentiation and supports the survival of neuroblasts derived from the neural crest in early development (Zhou XF 1998). The differentiation and survival of a subpopulation of large sensory neurons after their axons arrive at their targets is also dependent on presence of NT3 (Zhou XF 1998) and sympathetic neurons require NT3 for survival in the late developmental period (Tafreshi AP 1998). NT3 binds to all Trk receptors and some of its biological effects can occur through binding to Trks other than TrkC, which is its specific receptor. For instance the sympathetic neurons of the superior cervical ganglion require both NT3 and NGF for survival (Glebova NO 2005).

NT4 is the most recently discovered NT in mammals (Hallbook F 1991, IP NY 1992) and its biological role is not fully understood. NT4 has not been detected in avian species. All NT knockout mice have proven lethal during early postnatal development apart from NT4 deficient mice that only show minor cellular deficits and develop normally to adulthood. NT4 knockout mice have recently been reported to require NT3 in early postnatal development and NT4 later in mature animals for survival of sensory neurons. NT-4 infusion into the visual cortex alters responses and blocks the effect of monocular deprivation in cat during the critical period (Gillespie DC 2000). Even after responses to the deprived eye are adversely affected, NT-4 infusion is able to restore them (Gillespie DC

2000). These findings suggest that activation of NT-4 receptors during the critical period promotes connectivity independent of correlated activity.

All the NTs are synthesized as precursor proteins with an approximate molecular weight of 30 kDa. They are later cleaved to form the mature form and are all capable of forming noncovalent-linked homodimers with each other. Each monomer is approximately 13 kDa (reviewed by Huang EJ and Reichardt LF 2001).

The structures of NGF, NT-3, and NT-4 and of NT-3/BDNF and NT-4/BDNF dimers have been defined. A feature of their structures (a tertiary fold and cystine knot) is also present in several other growth factors, including platelet-derived growth factor (PDGF) and transforming growth factor β (TGF- β) (McDonald NQ 1991, Fandl JP 1994, Robinson RC 1995, 1999, Butte MJ 1998; reviewed in McDonald NQ & Chao MV 1995).

In human retina the exact cellular source of NT production and their receptors has not been described. In other mammals, NT production has been identified in retinal ganglion cells, amacrine cells and Müller cells (Garcia M 2003, Avwenagha O 2006).

The first suggestion of the clinical use of neurotrophins was in 1980s based on the observation that the basal forebrain cholinergic neurons are consistently affected at an early stage of Alzheimer's disease (Hefti F and Weiner WJ 1986). It has been shown that NGF is capable of preventing the degeneration of cholinergic neurons in adult rats with experimental lesions mimicking the cholinergic deficit in Alzheimer's (Hefti F and Weiner WJ 1986). Fisher *et al* have shown that in adult rats a continuous intraventricular infusion of NGF over a period of four weeks can partly

reverse the cholinergic cell body atrophy and improve retention of a spatial memory task in behaviourally impaired animals (Fischer W 1987). Several clinical trials have been conducted aiming to treat neurodegenerative disorders in which specific group of neurons are affected, as in amyotrophic lateral sclerosis, diabetic neuropathy, Alzheimer's and Huntington diseases, with disappointing outcomes (reviewed in Thoenen H & Sendtner M 2002). It remains to be seen if use of NTs in human retinal disorders has any beneficial effect.

1.6 Neurotrophin receptors

NTs exert their biological effects via two different classes of receptors. The low affinity receptor p75 bind all members of NTs. This receptor belongs to the tumour necrosis factor (TNF) receptor superfamily (Chao MV 1994, Bothwell M 1995). The cytoplasmic domain of this receptor contains a "death" domain structurally similar to those in other members of this receptor family (Liepinsch E 1997). p75 transmits signals important for determining appropriate apoptosis of neonatal neurons not reaching their proper targets (Nykjaer A 2005). During normal development nerve growth factor seems to be able to activate this low affinity receptor causing the death of neurons that lack TrkA (Dechant G 1997). Nerve growth factor can therefore prevent apoptosis through its specific receptor, trkA, but promote programmed cell death by signalling through p75 receptor (Dechant G 1997).

The high affinity receptors however belong to the tyrosine kinase receptor family. NGF binds to tropomyosin-related kinase A (TrkA), BDNF and NT4 both bind to TrkB and NT3 bind to TrkC.

NT binding to high affinity receptors leads to receptor dimerisation and subsequently activation of the tyrosine kinase present in their cytoplasmic domains. Phosphorylation of the intracellular catalytic domain will in turn initiate a phosphorylation cascade and cell growth or survival.

Neurotrophin	Specific Receptor	<i>Human chromosome location</i>
NGF	TrkA	<i>1</i>
BDNF	TrkB	<i>11</i>
NT3	TrkC	<i>12</i>
<i>NT4/5</i>	<i>TrkB</i>	<i>19</i>

Table 1.3 Chromosomal localisation of genes encoding for neurotrophins and their receptors.

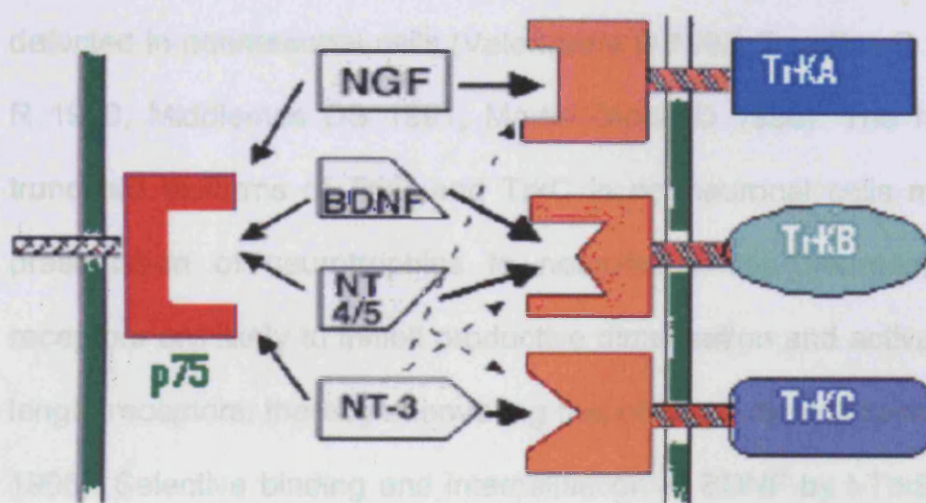


Figure 1.3 Diagramatic presentations of neurotrophic factors and their receptors.

NGF= Nerve growth factor, BDNF= brain derived neurotrophic factor,
 NT3= neurotrophin 3, NT4/5= neurotrophin 4/5, TrkA= tyrosine kinase A,
 TrkB= tyrosine kinase B, TrkC= tyrosine kinase C, p75=low affinity
 neurotrophin receptor

Binding of NTs to these truncated receptors (reviewed in Reichardt LF and Fariñas I 1997) does not cause activation of the intracellular pathway, for which they may function as dominant negative isoforms. Both full length and truncated form of TrkB and TrkC receptors have been detected in neurons while only truncated TrkB and TrkC isoforms have been detected in nonneuronal cells (Valenzuela D 1993, Tsoulfas P 1993, Klein R 1990, Middlemas DS 1991, Martin-Zanca D 1990). The functions of truncated isoforms of TrkB and TrkC in nonneuronal cells may include presentation of neurotrophins to neurons. Within neurons, truncated receptors are likely to inhibit productive dimerisation and activation of full-length receptors, thereby attenuating responses to neurotrophins (Eide FF 1996). Selective binding and internalisation of BDNF by t-TrkB has been reported suggesting that a function of this receptor might be to restrict BDNF availability. This may allow several BDNF sensitive systems to develop independently even though they are separated by only short distances (Biffo S 1995).

The distribution of TrkA and TrkB was first described in the ganglion cell layer, Müller cells, and optic axons of rat retina (Jelsma TN 1993, Zanellato A 1993). These receptors have also been reported to immunolocalise in all retinal cell layers in rat except the photoreceptor layer and optic nerve (Rickman DW 1995).

Expression of mRNA for all high affinity receptors has been detected in the neuroblastic inner nuclear and ganglion cell layers of embryo, newborn and adult rat retinas (Ernfors P 1992, Jelsma TN 1993). In chicks, TrkC

mRNA has been identified in all cell nuclear layers but predominates in the ganglion cell and inner nuclear layers (Bovolenta P 1996, Hallbook F 1996). In mammals the low affinity receptor, p75, has been found in Müller cells and retinal ganglion cells (Garcia M 2003, Schatteman GC 1988).

The expression of specific receptors for BDNF and NT4 by photoreceptor cells in mammals has not been demonstrated as yet and it is believed that protection of photoreceptors by neurotrophic factors occurs indirectly through activation of other cells (Wahlin KJ 2000). In their study Wahlin *et al* demonstrated that intravitreal injection of BDNF, CNTF and fibroblast growth factor (FGF2) in mice resulted in activation of intracellular signalling pathways in Müller cells, amacrine and ganglion cells. The same effect in signalling pathways was not demonstrated in the photoreceptor cells supporting the hypothesis that the effect of neurotrophic factors on the photoreceptors may be indirect through activation of Müller cells and other retinal cells (Wahlin KJ 2000).

In experimental studies in the rat, central nervous system injuries to the spinal cord or the brain are followed by down-regulation of neuronal Trk receptors (Kobayashi NR 1997, Liebl DJ 2001, Venero JL 1994). BDNF mRNA levels, however, are transiently up regulated after optic nerve crush (Gao H 1997) although this does not prevent or delay retinal ganglion cell death. The effect of BDNF injection or gene delivery to the retina is therefore expected to be short lived or even absent, as indeed is the case (Mansour-Robaey S 1994, Clarke DB 1998, Di Polo A 1998).

Cheng *et al* (Cheng L 2002) have demonstrated down-regulation of TrkB mRNA following optic nerve injury in rats and have further shown that

TrkB gene delivery in combination with exogenous BDNF extends the survival of retinal ganglion cells after injury.

1.7 Mechanism of intracellular message transmission

Ligand binding of TrkB receptors is known to result in phosphorylation of cytoplasmic tyrosine kinase residues on the cytoplasmic domain of these receptors. This autophosphorylation is then followed by downstream signal transduction of molecules such as mitogen activated protein kinase (MAPK) and cAMP responsive element binding protein (CREB) (Weng G 1999, Segal RA 1996, Meyer-Franke A 1995). The immediate early genes *c-fos* and *c-jun* are also rapidly upregulated in the activated cells (Sheng M 1990).

Recent reports studying the response produced via TrkB receptor after NT's binding suggest that BDNF and NT4 at very low concentrations (thousand-fold lower concentration than glutamate) are capable of depolarization of neurons in the central nervous system as rapidly as glutamate (Kafitz KW 1999). The same study reported that NT3 was found to produce much smaller response and NGF was ineffective.

It has been shown that in the rabbit retina BDNF can, within minutes, increase the release of dopamine from amacrine cells by activating TrkB receptors. The intracellular mechanism of TrkB receptor activation involves activation of phospholipase-Cgamma, with the subsequent production of inositol (1,4,5) trisphosphate (IP-3) and calcium release from the endoplasmic reticulum. This calcium release seems to be the actual trigger for BDNF-induced release of dopamine (Neal M 2003).

Studies using primarily PC12 cells have identified the key factors in the signalling pathways following binding of neurotrophins to Trk receptors. Based on studies involving Trk A receptor and NGF the following general scheme of intracellular signalling has been proposed (Kaplan D R & Stephens R M 1994). Binding of NGF to TrkA activates its tyrosine kinase activity, leading to phosphorylation on specific tyrosine residues within the intracellular domains of the receptor²⁰. These phosphotyrosyl residues act as docking sites for other signaling molecules, including phospholipase C- γ (PLC- γ) and Shc (Segal RA & Greenberg ME 1996, Kaplan D R & Miller FD 2000). Active PLC- γ cleaves phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) to generate both IP₃, and diacylglycerol. Additionally, tyrosine phosphorylation of Shc triggers Shc/Grb2/Sos interaction, Ras activation and a series of phosphorylation reactions including Raf, MEK and mitogen associated protein kinase (MAP kinase). Shc can also activate phosphoinositide 3-kinase (PI3 kinase) via protein-protein interaction involving Ras and Gab-1. PI3 kinase phosphorylates the phosphatidylinositol lipids to produce PI(3,4)P₂ and PI(3,4,5)P₃ (Toker A & Cantley LC 1997). In summary, binding of NGF to TrkA may activate at least three main signaling pathways in PC12 cells: PLC- γ , MAP kinase and PI3 kinase (Figure 1.4).

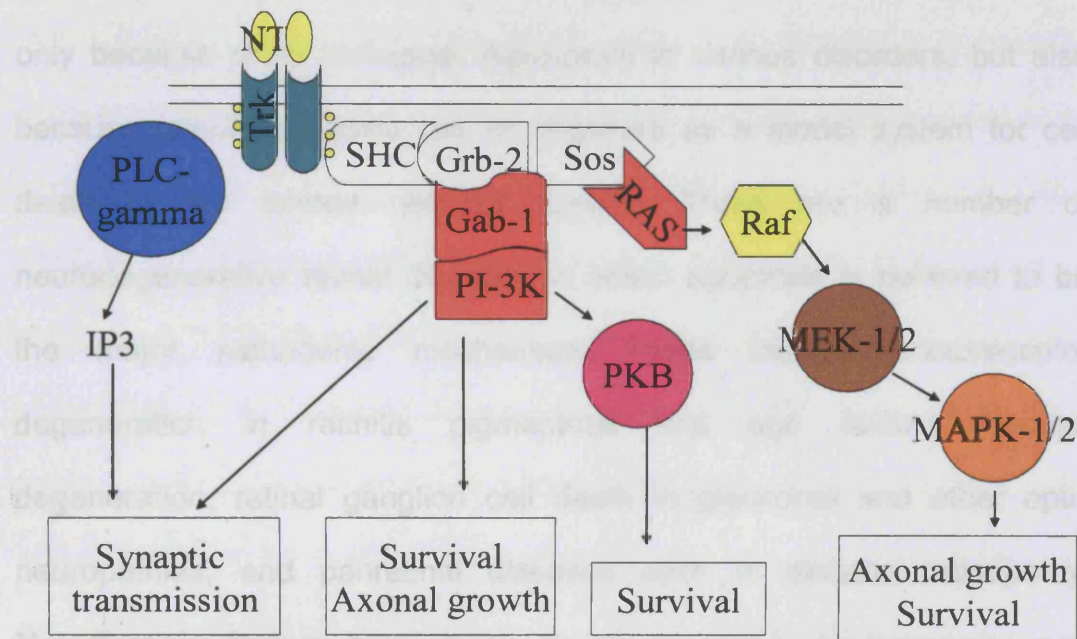


Figure 1.4 Intracellular signalling pathways associated with NT binding to Trk receptors. (Adapted from Thoenen H & Sendtner M 2002)

NT= Neurotrophins, Trk=Tyrosine kinase receptor, PLC-gamma= phospholipase C-gamma, IP3=inositol (1,4,5) trisphosphate, SHC= A transforming protein, Grb-2=Growth factor receptor bound protein 2, Sos= Son of sevenless protein, Gab-1= Grb-2-associated protein 1, PI-3K= phosphatidylinositol-3 kinase, PKB= Protein kinase B also known as AKT, RAS= RAS is a plasma membrane associated guanine nucleotide binding protein, Raf= Raf is a serine/threonine kinase normally positioned downstream of Ras in numerous signal transduction pathways, MAPK-1/2= Mitogen activated protein kinase, MEK-1/2= MAPK kinase.

1.8 Role of Müller cells in neuronal protection

Neuronal cell death in the retina is of special interest to investigators not only because of its biological importance in various disorders, but also because retinal apoptosis can be regarded as a model system for cell death in the central nervous system. There are a number of neurodegenerative retinal diseases in which apoptosis is believed to be the major pathogenic mechanism. These include photoreceptor degeneration in retinitis pigmentosa and age related macular degeneration, retinal ganglion cell death in glaucoma and other optic neuropathies, and panretinal diseases such in diabetic retinopathy. Neurotrophic factors have been shown to inhibit photoreceptor and ganglion cell apoptosis, both *in vitro* and *in vivo* (Steinberg RH 1994, LaVail MM 1998) but it is still unclear as how NTs protect photoreceptor cells from degeneration. Furthermore the expression of TrkB (specific receptors for BDNF) by photoreceptors in mammals has not been demonstrated as yet. It has therefore been proposed that NTs effect may be indirect via the supporting cells of the retina.

Müller cells, as the main glial element of the retina, express receptors for most of the molecules that have been shown to rescue photoreceptors. They also express glial fibrillary acidic protein (GFAP) in response to a number of stimuli including retinal degeneration (Ekstrom P 1988). It has been shown that loss of Müller cells can lead to photoreceptor death (Dubois-Dauphin M 2000). Whalin *et al* have shown that intraocular administration of BDNF can lead to activation of Müller cells and not photoreceptors (Wahlin KJ 2000).

The mechanism by which Müller cells can protect photoreceptors is however unclear and all the evidence is indirect. Release of a secondary factor by glial cells or a cell contact-mediated mechanism is a possible method of protection provided by these cells. Harada T et al (2000) have shown that basic fibroblast growth factor (FGF2), which is photoreceptive protector, is released at higher levels by Müller cells in response to exogenous NT3. They have also demonstrated that the reverse is true when NGF is administered causing a decrease in FGF2 production as a result. There is therefore a fine balance between pro and anti apoptotic effect that can be initiated by Müller cells in response to exogenous neurotrophic factors.

The low affinity NT receptor, p75, is expressed in human Müller cells (Garcia M 2003) and its expression is up regulated in diabetes (Hammes HP 1995). Ischemia and reperfusion in rats' retina is also associated with up-regulation of p75 (Tomita H 1998, Vecino E 1998) suggesting an enhanced role by Müller cells in response to available neurotrophins and therefore increased neuroprotective effect (Vecino E 1998).

1.9 Neurotrophins in retinal laser injury

Laser photocoagulation is an established treatment for a number of retinal neovascular diseases, however the mechanism of its action is not yet fully understood. Retinal photocoagulation causes outer retinal necrosis, hyperplasia of the RPE and glial cell proliferation and migration, eventually leading to localized fibrous scar formation (van der Zypen E 1986, Marshall J and Bird A 1979, Tobe T 1995). Cellular activation may be reflected in changes of expression of various markers such as glial

fibrillary acidic protein (GFAP), which is highly expressed by astrocytes but minimally expressed by resting Müller cells, which increase their expression of GFAP when activated (Humphrey MF 1997). Similarly, RPE cells, which do not normally stain for CD68 *in vivo*, express this molecule under culture conditions as evidence of activation (Limb GA 1997). Up-regulation of cell marker expression has become an important marker of injury in central nervous system tissues including the retina (Humphrey MF 1997, Lewis GP 1989), where GFAP up regulation in Müller cells occurs rapidly after retinal detachment (Erickson PA 1987) as well as following laser photocoagulation (Humphrey MF 1993 and 1997). The cellular and molecular events induced by retinal photocoagulation and the subsequent wound healing responses are complex and as yet incompletely characterized processes. Experimental models suggest that these events are regulated by a variety of growth factors (Xiao M 1999). Changes in pattern of staining for NT4, GFAP and CD68 have been reported following laser photocoagulation in the human retina (Ghazi-Nouri S 2003).

1.10 Investigation of the neuroprotective role of Müller glia in the retina

There is sufficient evidence that glial cells play a central role in the nervous system through their release of neurotrophins, as that illustrated by Müller glia in the retina. Although several studies have examined the role of Müller glia in different animal species, there is only one published report investigating the mRNA expression of NGF, BDNF and NT3 and their specific receptors by cultured human Müller cells (Oku H 2002). This

study did not investigate the mRNA expression of NT4 and did not examine their protein expression by Western blot analysis.

To investigate the role of these molecules in the pathogenesis of retinal degenerative disease, it is therefore of interest to investigate the expression of these growth factors as well as their receptors *in vitro* at mRNA and protein level using cultured human Müller cells. It is also of particular importance to study whether changes in expression of neurotrophins and their receptors *in vivo* may be observed in conditions such as PVR and laser induced retinal injury. This would provide better understanding of the role of Müller cells and neurotrophins in retinal neuroprotection. To investigate the expression of these molecules, a series of laboratory experiments and clinical studies were designed with the following aims:

Aims

The aims of the work in this thesis were to:

- Characterise the expression of BDNF and NT4 and their common receptor, TrkB, by cultured human Müller cells at mRNA and protein levels.
- Define the expression of BDNF, NT4 and TrkB in normal human retina.
- Investigate the changes in expression of BDNF, NT4 and TrkB in retinectomy sections of patients with PVR.
- Determine the changes in expression of BDNF, NT4 and GFAP in melanoma affected human retina following laser photocoagulation.

Chapter 2

Materials and Methods

2.1 Cell line and culture conditions

MIO-M1: Moorfields, Institute of Ophthalmology-Müller 1 cell line (Human Müller cell line).

MIO-M1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Lois, MO) containing glutamax-1, sodium pyruvate, 4500mg/l glucose and pyridoxine. All media were supplemented with 10% foetal calf serum (FCS), penicillin (42U/ml), streptomycin (42 U/ml) and incubated at 37°C with 5% CO₂. Cells were kept sub-confluent and passaged every 3-4 days. For passage, they were washed twice in phosphate buffered saline (PBS) before being harvested using Trypsin-EDTA and then washed in DMEM and re-plated. Subcultures 20 to 30 were used in the experiments.

2.2 Cell culture with PDGF supplement

Cells were incubated under conditions in section 2.1 for 24, 48 and 72 hours. Platelet derived growth factor (PDGF) (Sigma, St. Lois, MO) in a total concentration of 50 ng/ml (Haber M 2003, Price RL 2003, Zheng Y 2003) was added to culture medium and RNA was isolated 24 hours later to assess the effect of PDGF on mRNA expression in these cells.

2.3 Cell culture with NT4 supplement

To assess the effect of NT4 on mRNA expression of t-TrkB MIO-M1 cells were cultured as above and recombinant human NT4 (R&D Systems, Mineapolis, MN, USA) was added at concentrations of 1ng/ml, 10ng/ml, or 50ng/ml to the culture medium every day for 5 days prior to RNA extraction for RT-PCR.

2.4 Preparation of cell lysates for Western blotting

Cell lysates were prepared using sub-confluent cultures. Medium was removed and cells were washed twice in PBS. Cells were harvested using Trypsin-EDTA, washed in medium and spun for 5 minutes to form a pellet. This was washed in PBS and lysed with 200ul cold radioimmune precipitation assay buffer (RIPA buffer: 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 158 mM NaCl, and 50 mM Tris [pH 7.2]). Samples were stored at -80°C until use.

2.5 Western blot

Expression of BDNF, NT4 and TrkB proteins by cultured MIO-M1 cells was assessed using Western blot analysis. Aliquots of Müller cell lysates (1.5 mg/mL, measured using spectrophotometer, Eppendorf BioPhotometer, Hamburg, Germany) were resolved on 7% Tris-acetate polyacrylamide gels (NuPAGE; Invitrogen, Groningen, The Netherlands) for 50 minutes at 2000 V in Tris-acetate running buffer (50 mM Tris, 50 mM tricine, and 0.1% SDS [pH 8.3]; Invitrogen, Paisley, UK). Molecular weight marker (Santa Cruz, Ca, USA) was used as internal standards. Proteins were then transferred to nitrocellulose membranes for 60 minutes at 30 V followed by incubation in 2% blocking reagent (in TBS, pH 7.4) for 2 hours at room temperature. Membranes were then incubated with primary rabbit polyclonal antibody (Santa-Cruz, 1 in 200 dilution) overnight. Membranes were washed in Tween TBS 0.1% twice and incubated with HRP conjugated goat anti-rabbit IgG (Santa Cruz) for 90 minutes (1 in 1500 dilution).

Immunocomplexes were detected by enhanced chemiluminescence (ECL; Amersham, Amersham, UK). Images were analyzed and processed using an image reader (LAS-1000 Pro, ver. 2.1; Fuji, Bedford, UK).

Mouse brain extract (Santa Cruz, Ca, USA) was used as positive control for BDNF and NT4 antibodies.

2.6 Antibodies and dilutions used for Western blotting

All antibodies were supplied by Santa Cruz (Santa Cruz, Ca, USA) and were polyclonal rabbit antibodies. Dilutions used were 1:200 in each case (Table 2.1) as recommended by the supplier.

Primary antibodies	species	dilution
Anti BDNF (Santa-Cruz)	rabbit polyclonal	1:200
Anti NT4 (Santa-Cruz)	rabbit polyclonal	1:200
Anti TrkB (Santa-Cruz)	rabbit polyclonal	1:200
Anti t-TrkB (Santa-Cruz)	rabbit polyclonal	1:200

Table 2.1 Antibodies and dilutions used for Western blotting

2.7 RNA extraction

Müller cells were cultured as described above. An RNA isolation kit (RNeasy, Qiagen, West Sussex, UK) was used for RNA extraction according to the protocol. Cells were trypsinised followed by addition of DMEM plus 10% FCS medium to inactivate the trypsin. Cells were subsequently centrifuged at 300 x g for 5 minutes and the supernatant was completely aspirated. The pellet obtained was re-suspended in the

lysis buffer containing guanidine isothiocyanate (GITC) and β -Mercaptoethanol (β -ME) followed by passing the lysate at least 5 times through a 20-gauge needle fitted to an RNase-free syringe. Ethanol (70%) was then added to the sample and it was applied to an RNeasy mini column for RNA to bind to the membrane and contaminants were washed away. The bound RNA was eluted in 30 μ l of water and the RNA concentration was quantified using Eppendorf Biophotometer (Eppendorf, Hamburg, Germany) prior to storing at -80°C till use.

2.8 RT-PCR

cDNA samples were prepared by using mRNA transcription Promega Reverse Transcriptase System (Promega, Southampton, UK). Gene specific primers were used as shown in Table 2.2.

For each PCR, cDNA corresponding to 0.1 μ g RNA was amplified in a reaction tube of total volume 100 μ l containing 200 μ M dNTPs, 2mM MgCl_2 (with contribution from first-strand cDNA reaction), 10mM Tris-HCl, 50mM KCl, 0.1% Triton X-100, 1 μ l of each upstream or downstream primer and 2.5 unit of *Taq* DNA polymerase. A reaction tube containing GAPDH primers was used as internal control for the PCR reactions. Two negative controls were included; one including all PCR reaction components except primers and another one with 0.1 μ g RNA instead of cDNA to ensure the absence of residual DNA contamination. GAPDH primers were used in this negative control reaction tube. PCR cycles consisted of an initial 2 minute denaturation step at 94°C followed by 35 cycles of 94°C for 30 seconds, 60°C for 1 minute, 68°C for 2 minutes, and a final extension at

68°C for 7 minutes in an automated thermal cycler (Mastercycler gradient, Eppendorf, UK).

2.9 PCR primers

Gene specific primers were used for RT-PCR experiments to amplify BDNF, NT4, TrkB, t-TrkB and GAPDH. The primer used for TrkB amplification was specific for the kinetic domain of the receptor, recognising the full-length receptor. The full sequence of the gene for these factors is in the appendix.

Gene	Size (bp)	Upstream sequence (5'- 3')	Downstream sequence (5'- 3')
BDNF	222	AAC AAT AAG GAC GCA GAC TT	TGC AGT CTT TTT GTC TGC CG
NT4	202	AGC GAA ACT GCA CCA GCG AG	CAC CTT CCT CAG CGT TAT CA
TrkB (kin)	571	AGG GCA ACC CGC CCA CGG AA	GGA TCG GTC TGG GGA AAA GG
t-TrkB	161	TAA AAC CGG TCG GGA ACA TC	ACC CAT CCA GTG GGA TCT TA
GAPDH	600	CCA CCC ATG GCA AAT TCC ATG GCA	TCT AGA CGG CAG GTC AGG TCC ACC

Table 2.2 Gene specific primers used in RT-PCR to detect BDNF, NT4, TrkB kinetic domain for the full-length receptor, t-TrkB and GAPDH.

2.10 Horizontal agarose gel electrophoresis

Amplified cDNA samples were resolved in 1% agarose gel containing 2 μ l ethidium bromide (10mg/ml stock solution) per 100 ml gel. The gel was immersed in 1X TAE buffer and 100V was applied for approximately 1 hour. A 100bp DNA ladder (New England Biolabs, Ipswich, MA, USA) was used as marker and 6X loading dye (Fermantas, Ontario, Canada) added to each sample prior to loading into the gel.

2.11 Preparation of cells for immunocytochemistry

Müller cells were cultured for 48 hours on fibronectin-coated (5 μ g/mL) glass chamber slides (NalgeNunc, Inc., Roskilde, Denmark), fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.2) for 10 minutes, and incubated for 1 hour with primary antibodies (see antibodies and dilutions as listed in table 2.1) diluted in 0.5% blocking reagent (Roche Molecular Biochemicals, Lewes, UK) in Tris-buffered saline (TBS; pH 7.5).

For negative controls, rabbit IgG isotypes (DAKO, Glostrup, Denmark) matching those of the test antibodies were used at the same concentrations as above. A second negative control contained 1X TBS instead of the primary antibody. After incubation with primary antibody, specimens were washed in TBS, followed by incubation for 45 minutes with goat anti-rabbit antibodies conjugated with FITC (Alexa Fluor 546, see table 2.3) (Invitrogen, Paisley, UK). Specimens were then washed and mounted on glass slides (Vectashield mounting medium; Vector Laboratories, Burlingame, CA). Fluorescent images were recorded using a confocal scanning laser microscope (BioRad Radiance 2000 AGR-3 (Q)).

2.12 Tissue preparation for immunohistochemistry

Human retinal tissues were fixed in 4% paraformaldehyde (4%PFA) and kept at 4° C until use. PVR retinectomy specimens were fixed immediately in operating theatre. The cadaveric donor tissues were fixed within 24 to 48 hours from harvesting. The PFA was replaced with fresh solutions once a month. Embedding medium consisted of 5g agarose type XI of low gelling temperature (Sigma A3038) and 1g sodium azide in distilled water in a total volume of 100ml. This mixture was heated at approximately 50° C.

Specimens were washed in 1X PBS buffer prior to being inserted and orientated in the agarose solution. The gel was allowed to cool and solidify and stored within a humidity box at 4°C till ready for sectioning.

Vibratome series 1000 (Agar Scientific, Essex, UK) was used for sectioning the specimens with low speed, the amplitude set on 5 and the thickness on 100 microns. Sections were stored in 4% PFA prior to staining.

For immunostaining specimens were placed in Multidish wells (NalgeNunc, Inc., Roskilde, Denmark) free floating in 500 µl 1XPBS for ½ hour and then incubated with primary antibodies (see table for details and dilution) at 4°C on a shaker overnight. After a further period of ½ hour wash in 1XPBS, secondary antibodies (see table for details and dilutions) were added to the wells and incubated again overnight 4°C on a shaker. To-Pro3-Iodide (1:5000 dilutions) was used for 1 hour for nuclear staining. Specimens were washed as above prior and after nuclear staining.

Rabbit IgG (DAKO, Golstrup, Denmark) and Mouse IgG (Sigma, St. Lois, MO, USA) were used at the same concentration as the primary antibodies for negative controls. A second negative control was also prepared without addition of primary antibodies.

Specimens were then washed and mounted on glass slides (Vectashield mounting medium; Vector Laboratories, Peterborough, UK). Fluorescent images were recorded using a scanning laser confocal microscope (BioRad radiance 2000 AGR-3 (Q)).

Primary antibodies	Species	Dilution
Anti BDNF (Santa-Cruz)	rabbit polyclonal	1:200
Anti NT4 (Santa-Cruz)	rabbit polyclonal	1:200
Anti TrkB (Santa-Cruz)	rabbit polyclonal	1:200
Anti t-TrkB (Santa-Cruz)	rabbit polyclonal	1:200
Anti-Rhodopsin (Rho4D2) (gift from Bob Molday, Canada)	mouse monoclonal	1:40
GFAP (Santa-Cruz)	mouse monoclonal	1:200
Secondary antibodies	Species	Dilution
Anti-rabbit (Alexa Fluor 546)	goat	1:500
Anti-mouse (Santa-Cruz)	donkey	1:250
Anti-mouse (Alexa Fluor 488)	donkey	1:500

Table 2.3 Primary and secondary antibodies used in double-labeling of cells and retinal tissues.

2.13 Confocal laser scanning microscopy of fixed Müller cells and retinal tissues

A BioRad Radiance 2000 AGR-3 (Q) laser scanning confocal microscope was used for imaging of stained Müller cells and retinal tissues. A series of images through the thickness of the specimens were obtained and stored for image analysis. Images were analysed by three different observers and there was complete agreement in their findings during image analysis.

2.14 Laser induced injury in patients

2.14.1 Patients and methods

Laser photocoagulation was performed on the retinas of 4 patients (2 males and 2 females, median age 70 years) prior to undergoing enucleation for malignant melanoma of the choroid (Table 2.4). The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and approved by Moorfields Eye Hospital Research Ethics Committee.

Patients with pre-existing retinal disease or clinically significant retinal detachment associated with the choroidal melanoma were excluded from the study. All four patients received three rows of 10 - 15 creamy white argon green laser burns away from the choroidal melanoma. Laser photocoagulation was performed 1 day prior to enucleation in one patient, 2 days prior to enucleation in one patient and 6 days prior to enucleation in 2 patients. Laser spots were 500 micron and of 0.1 second duration. Other laser treatment details are summarized in table I. Eyes were fixed in

4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) immediately after enucleation.

2.14.2 Immunohistochemical staining of lasered retina

Retinal tissue containing the laser foci were paraffin embedded and sections of 7µm thickness prepared for immunohistochemistry. Retinal sections from three non-lasered eyes with malignant choroidal melanoma and three normal eyes from corneal donors with normal retina were used as controls. Slides were immersed in pepsin solution (0.4 gms in 100 mls of 0.1N HCL) at 37°C for 15 minutes, washed twice in tris buffered saline (TBS) and placed in 0.5% blocking reagent (Roche Molecular Biochemicals, Lewes, UK) in TBS for 20 min. Sections were incubated overnight at room temperature with a panel of primary monoclonal antibodies diluted in 0.5% blocking reagent in TBS as follows: Anti- BDNF (MAB248, 10ug/ml- R&D Systems, UK); anti-NT3 (MAB267, 5ug/ml- R&D Systems, Mineapolis, MN, USA); anti-NT4 (MAB268, 5ug/ml- R&D Systems, Mineapolis, MN, USA); anti-GFAP (Clone 6F2- 10 ug/ml- Dako, Glostrup, Denmark) and anti-CD68 (Clone PG-M1 10ug/ml- Dako, Glostrup, Denmark). Mouse IgG isotypes matching those of the test antibodies (Sigma, St. Lois, MO, USA) were used as negative controls in the assay. Following incubation with primary antibody, specimens were washed and further incubated for 30 min with 50 µl of biotin-labelled rabbit anti-mouse antibody (Dako, Glostrup, Denmark). After washing in TBS, slides were then covered with 50 µl Strept/Avidin/AP complex (Dako, Glostrup, Denmark) and incubated for 45 minutes, washed twice with TBS and further incubated for 30 min with 50 µl of alkaline phosphate substrate

(Vector Red- Vector Laboratories, Peterborough, UK). Slides were counterstained with Mayer's haematoxylin prior to microscopy analysis. Selected non-immunolabelled sections were also stained with haematoxylin and eosin to permit evaluation of the overall morphology of the sections. Slides were analysed by three different observers under light microscopy and there was complete agreement in their findings during slide analysis.

Origin of retinal specimen (age, gender)	Location of laser burns (spot energy)	Laser prior to enucleation (days)
Melanoma (70,M)	Nasal (400-700mW)	1
Melanoma (82,F)	Superior (410-650mW)	2
Melanoma (70,F)	Inferior (30-60 mW)	6
Melanoma (47,M)	Superior (30-60 mW)	6
Melanoma (68, M)	No laser	n/a
Melanoma (70, M)	No laser	n/a
Melanoma (46, F)	No laser	n/a
Cadaveric donor (73, M)	No laser	n/a
Cadaveric donor (26, M)	No laser	n/a
Cadaveric donor (66, M)	No laser	n/a

Table 2.4 Details of specimen used in the laser induced injury study of retinal tissues.

All melanoma affected eyes were fixed in 4% paraformaldehyde (PFA) immediately after enucleation. The cadaveric donor eyes were fixed in 4% PFA within 24 to 48 hours from harvesting.

Sample	Age (years)	Gender	Condition
1	80	F	PVR
2	56	F	PVR
3	72	F	PVR
4	67	F	PVR
5	70	M	PVR
6	52	M	PVR
7	55	M	PVR
8	38	M	PVR
9	55	M	Cadaveric donor
10	50	M	Cadaveric donor
11	75	M	Cadaveric donor

Table 2.5 Details of specimens used in the immunohistochemistry study of retinal tissues.

The PVR specimens were fixed in 4% paraformaldehyde (PFA) immediately in theatre. The cadaveric donor tissues were fixed in 4% PFA within 24 to 48hours from harvesting.

Chapter 3

Results

3.1 Detection of BDNF protein in cultured Müller cell lysates

To determine the expression of BDNF at protein level in cultured human Müller cells, Western blot analysis of both lysates and supernatants of MIO-M1 cell line were performed. Cell lysates examined at 24, 48, and 72 after culture identified a band measuring approximately 40kDa (figure 3.1, lanes 2-4). The same band was also identified in the mouse brain extract (Santa Cruz Biotechnology, CA) used as positive control (figure 3.1, lane 9). This indicated presence of BDNF pro-protein (see discussion). Two other non-specific bands were also identified at approximately 90 and 100 kDa (figure 3.1, lanes 2-4 and 9). No bands were identified in the supernatants after 24 hours culture (figure 3.1, lane 5).

The expected size for BDNF monomer is approximately 14kDa but a band of this molecular weight was not detected in any of the Western blot experiments performed. Anti-BDNF antibody did not react with recombinant human NT-4 (lane 8) indicating no cross reactivity.

3.3 Western blotting: NT-4 in cultured MIO-M1 cell lysates and supernatants

These experiments were performed to detect NT-4 in cultured MIO-M1 cell lysates and supernatants after 24, 48 and 72 hours of culture. The results are presented in Figure 3.1. The Western blot analysis (not shown) in the Figure 3.2 presented here was not included in this study. There was also no detectable NT-4 in the supernatants of cell culture. Experiments performed under different conditions detected

the positive control protein (recombinant human NT-4, R&D Systems, Minneapolis, MN, USA) at appropriate expected size of 14 kDa. It also identified a slower migrating protein at approximately 28 kDa, possibly

Figure 3.1 Detection of BDNF by Western blot analyses of MIO-M1 cell lysates and supernatants after cell culture for various time periods.

Lane orders;

1= Molecular weight ladder (Santa Cruz Biotechnology, CA, sc-2035),

2= lysate prepared after 24 hours culture

3=lysate prepared after 48 hours culture

4=lysate prepared after 72 hours culture

5= 24 hours supernatant control,

6= 48 hours supernatant control,

7= 72 hours supernatant control,

8= Recombinant human NT-4,

9= Mouse brain extract,

10= Molecular weight ladder (Santa Cruz Biotechnology, CA, sc-2035)

Anti-NT4 antibody only reacted with the rNT4 used as positive control.

This antibody did not identify any proteins in cell lysates or supernatants.

3.2 Western immuno-blot to detect NT4 in cultured Müller cell lysates and supernatants

These experiments did not detect NT4 protein in cell lysates and supernatants after 24 hours (not shown in the figure 3.2 presented here but studied in several experiments) 48 and 72 hours. There was also no detectable protein in the supernatant after 72 hours of cell culture. Experiments repeated several times under standard conditions detected the positive control protein (recombinant human NT4, R&D Systems, Mineapolis, MN, USA) at appropriate expected size of 14 kDa. It also identified a slower migrating protein at approximately 28 kDa, possibly NT4 dimer (figure 3.2).

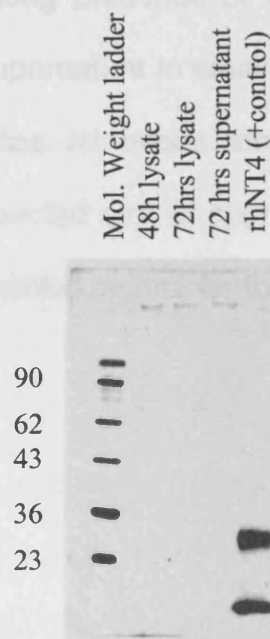


Figure 3.2 Western immunoblot to detect NT4 in cultured MIO-M1 cell lysates and supernatants.

Anti-NT4 antibody only reacted with the rhNT4 used as positive control. This antibody did not identify any proteins in cell lysates or supernatants.

3.3 Detection of Truncated TrkB in cultured Müller cell lysates

We next examined the expression of TrkB, the specific receptor for BDNF and NT4, by cultured MIO-M1 cells and analysed the supernatant and the lysates at various time points using Western blot technique. The antibody used (Santa Cruz, Biotechnology CA) reacts with both the full length and the truncated receptor as it targets the extracellular component of the receptor.

Two protein bands of similar size of approximately 80 kDa were detected at 96 hours after cell culture corresponding to the truncated isoform of the receptor (figure 3.3, lane 3). There was no clear band identifiable in the supernatants, however, a very faint trace could be seen at the same level as the lower band suggesting presence of the lower molecular weight truncated receptor in the supernatant in smaller quantities compared with that present in the cell lysates. No bands corresponding to the full-length receptor was identified (expected size of approximately 145 kDa). There was no suitable available positive control for this experiment.

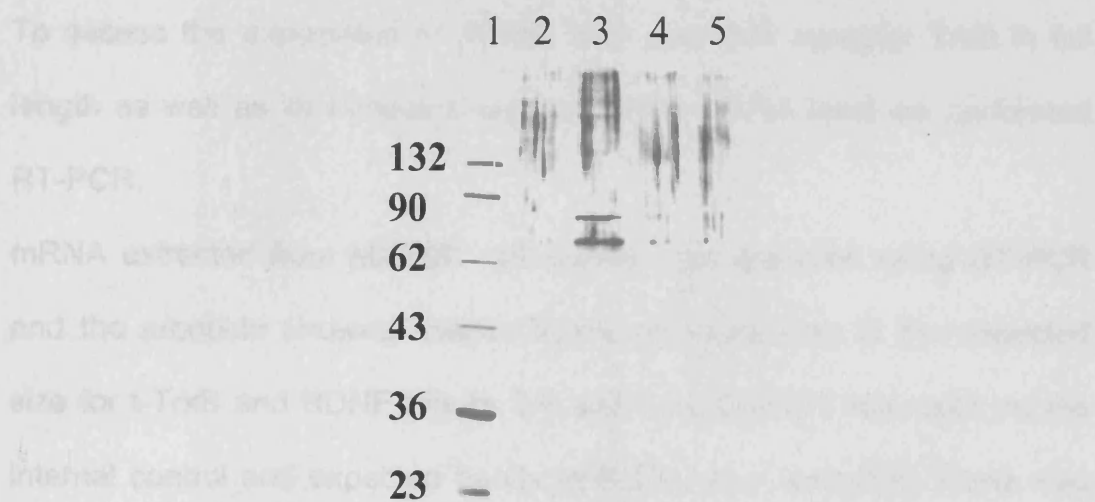


Figure 3.3 Detection of t-TrkB by Western blot analysis of MIO-M1 cell lysates and supernatants after cell culture at various time periods.

This figure shows that two strong and distinct bands were identifiable of approximately 80 kDa in the cell lysates following 96 hours of cell culture. Only a very faint band was detected in lysates prepared after 72 hours of cell culture as well in the supernatants after 96 hours. These bands would approximately correspond to the expected molecular size of truncated receptors.

Lane orders; 1= Molecular weight ladder (Santa Cruz, sc-2035), 2= Cell lysates after 72 hours of cell incubation, 3= Cell lysates after 96 hours of cell incubation, 4= Supernatants after 96 hours of cell incubation, 5= Negative control.

3.4 PCR amplification of BDNF, NT4 and t-TrkB

To assess the expression of BDNF, NT4 and their receptor TrkB in full length as well as its truncated isoform at the mRNA level we performed RT-PCR.

mRNA extracted from MIO-M1 cell lysates was analysed using RT-PCR and the products showed distinct bands corresponding to the expected size for t-TrkB and BDNF (figure 3.4 and 3.5). GAPDH was used as the internal control and expected bands of 600bp were amplified. There was no NT4 detectable using this set of primers.

A separate set of primers were subsequently used to detect BDNF and NT4 which successfully demonstrated expression of both of these growth factors at the RNA level (figure 3.6). The intensity of NT4 bands detected was uniform at 24 and 48 hours after cell culture. Similarly the band intensity corresponding to BDNF expression at 24, 48 and 72 hours suggested there was comparable amount of RNA expression at these time points. No bands were detected in negative controls.

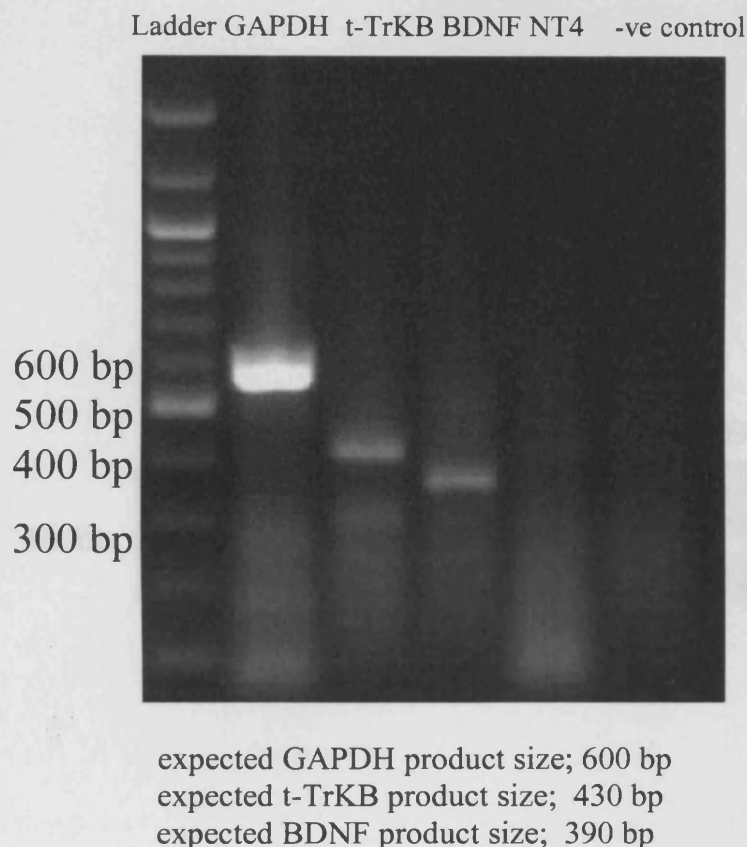


Figure 3.4 Detection of BDNF and t-TrkB by RT-PCR.

RNA specimens extracted from MIO-M1 cell lysates were analysed using RT-PCR and the products show distinct bands corresponding to the expected size for t-TrkB (lane 3) and BDNF (lane 4). GAPDH was used as the internal control and the expected band of 600bp was amplified (lane 2). There was no NT4 detectable using this method and no bands were detected in the negative control lane.

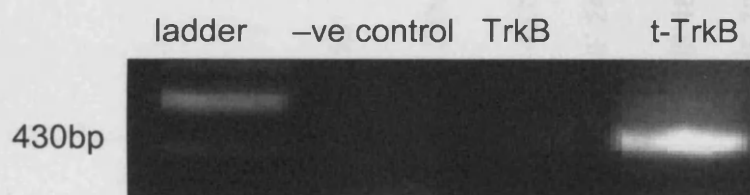


Figure 3.5 RT-PCR result demonstrating expression of t-TrkB mRNA by cultured MIO-M1 cells.

Expected product size of 430 bp was detected in lane 4 confirming mRNA expression of t-TrkB by cultured MIO-M1 cells. There was no detectable product for the full length receptor.

3.5 FGF2r supplement up-regulated the expression of BDNF in cultured MIO-M1 cells.

Addition of FGF2 to the culture medium (50ng/ml) led to an increase in intensity of the detectable band corresponding to BDNF expression (figure 3.7).

3.7. This did not affect the expression of NT-4. No RT-PCR products were detected in negative control lanes (figure 3.7 lanes 10 and 11).

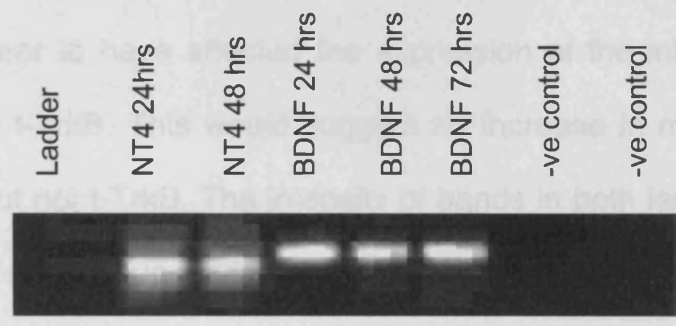
expression of BDNF and NT-4. The expected size of BDNF RT-PCR product was 222bp and NT-4 RT-PCR product was 202bp.

expression by addition of FGF2. There were no detectable bands corresponding to expected size for NT-4 expression. No RT-PCR products were detected in negative control lanes (figure 3.7 lanes 10 and 11).

11).

Figure 3.6 Detection of BDNF and NT4 in cultured MIO-M1 Müller cells by RT-PCR.

In this experiment a separate pair of primers to those used previously (in figure 3.4) was used to detect mRNA expression of BDNF and NT-4 by cultured Müller cells. Bands of 202bp corresponding to the expected size for NT4 RT-PCR product were detected in both lanes 2 and 3. Similarly, expected band size of 222 bp was detected in lanes 4-6 confirming mRNA expression of BDNF. There were no detectable bands in negative controls (lanes 7 and 8).



3.5 PDGF supplement up-regulates the expression of BDNF in cultured MIO-M1 cells

Additions of PDGF to the culture medium (50ng/ml) lead to an increase in intensity of the detectable band corresponding to BDNF expression (figure 3.7). This did not appear to have affected the expression of the internal control GAPDH or the t-TrkB. This would suggest an increase in mRNA expression of BDNF but not t-TrkB. The intensity of bands in both lanes 6 and 7 (figure 3.7) was similar suggesting no detectable effect on GAPDH expression by addition of PDGF. There were no detectable bands corresponding to expected size for NT-4 amplification. No RT-PCR products were detected in negative control lanes (figure 3.7 lanes 10 and 11).

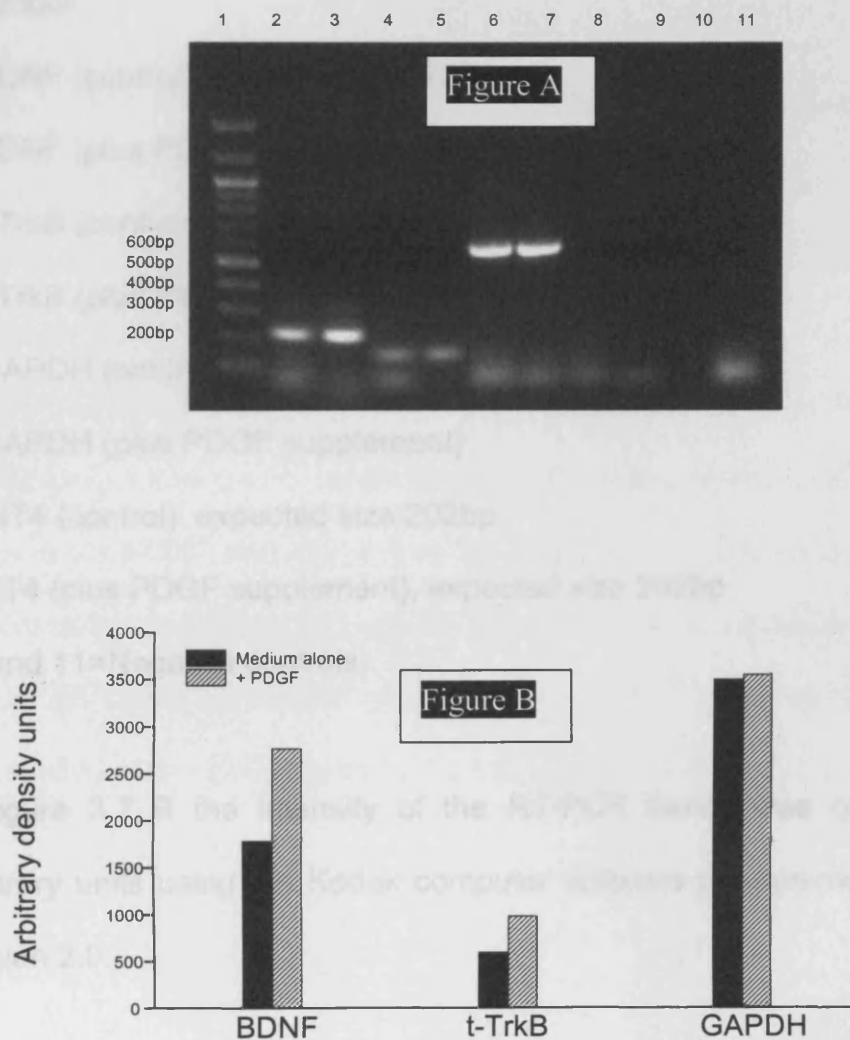


Figure 3.7, RT-PCR products showing the effect of PDGF (Sigma, 50 ng/ml) on BDNF and t-TrkB expression (figure A). Density measurements for RT-PCR products (figure B).

Addition of PDGF to the culture medium lead to an apparent increase in BDNF expression as evident by higher intensity band detected in lane 3 compared to that in lane 2. This did not appear to have affected the expression of the internal control GAPDH (lanes 6 and 7) or the t-TrkB (lanes 4 and 5).

Lane orders in figure 3.7 A;

1= ladder

2=BDNF (control), expected size 222 bp

3=BDNF (plus PDGF supplement), expected size 222bp

4=t-TrkB (control), expected size 161 bp

5=t-TrkB (plus PDGF supplement), expected size 161bp

6=GAPDH (control)

7=GAPDH (plus PDGF supplement)

8= NT4 (control), expected size 202bp

9=NT4 (plus PDGF supplement), expected size 202bp

10 and 11=Negative controls.

In figure 3.7 B the intensity of the RT-PCR bands was quantified in arbitrary units using the Kodak computer software programme KDS1D – version 2.0.

3.6 Phenotypic distribution of BDNF, NT4 and t-TrkB in cultured cells

We next examined the expression of BDNF in cultured MIO-M1 cells after fixing them in PFA to identify the pattern of distribution and localisation of this factor within the cells.

BDNF was detected within the cells and distribution was found to be mainly peri-nuclear with some protein detectable in the cell processes. Staining for BDNF showed a granular appearance (figures 3.8 panel A). NT4 protein expression had similar appearance and distribution as BDNF (figure 3.8 panel B). Protein expression of the truncated receptor, t-TrkB, was mainly at the peri-nuclear area and again had a granular appearance (figure 3.8 panel C).

3.7 Immunolocalisation of BDNF in the normal human retina

In order to localise BDNF staining in human retina, retinal donor eyes

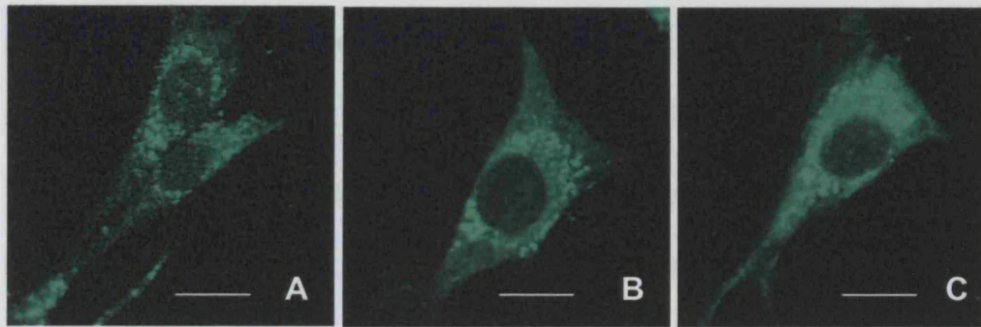


Figure 3.8 Scanning laser confocal image of cultured MIO-M1 cells stained with anti BDNF (panel A), anti NT4 (panel B) and anti t-TrkB (panel C) antibodies.

These images show immunostaining of BDNF, NT4 and t-TrkB with a granular appearance. Their distribution was mainly peri-nuclear with some protein staining detectable in the cell processes. Scale bars=20µm.

3.7 Immunolocalisation of BDNF in the normal human retina

In order to localise BDNF staining in human retina normal donor eyes retinas were examined and the peripheral retina was stained by immunohistochemical methods using anti BDNF antibody. Rods and nuclear layers were counter stained to identify the retinal structures and to identify any possible co-localisations. Scanning laser confocal images showed BDNF expression on the nerve fibre and the photoreceptor layers. The intensity of staining for this factor was generally weak. There was also co-localisation of BDNF and rhodopsin suggesting the presence of this growth factor in rods (figures 3.9). There was no detectable BDNF in the inner and outer nuclear layers. Figure 3.10 shows another normal retina demonstrating similar pattern of staining for BDNF in photoreceptor and nerve fibre layers.

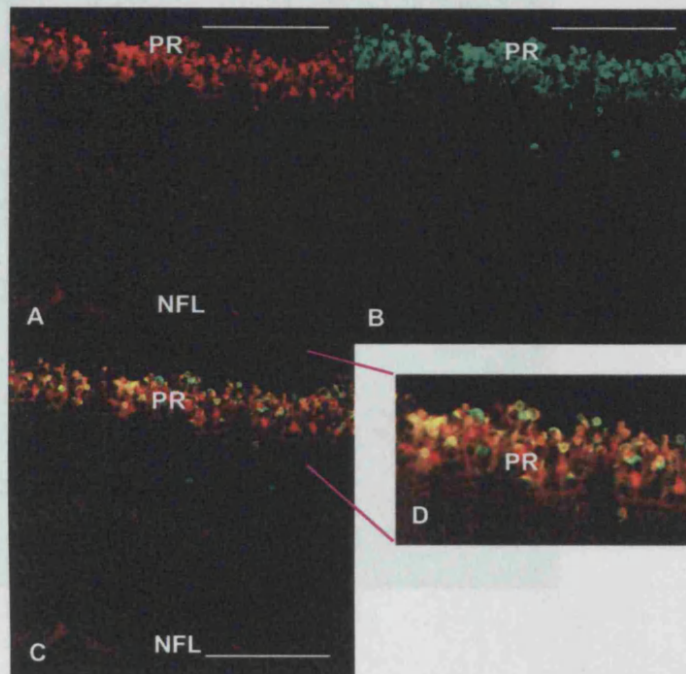


Figure 3.9 Double-labeled immunostaining using antibodies to BDNF (red) and rhodopsin (green).

Panel A shows pattern of BDNF staining. Panel B demonstrates staining of the photoreceptors (Rods). The merged image of A and B is shown in the panel C. Panel D shows the magnified section of photoreceptors. In normal human retinas there is expression of BDNF at the photoreceptor (PR) and the nerve fibre layers (NFL). Panels C and D demonstrate the co-localisation of BDNF with rhodopsins suggesting the presence of this growth factor in rods. Scale bars= 100μm.

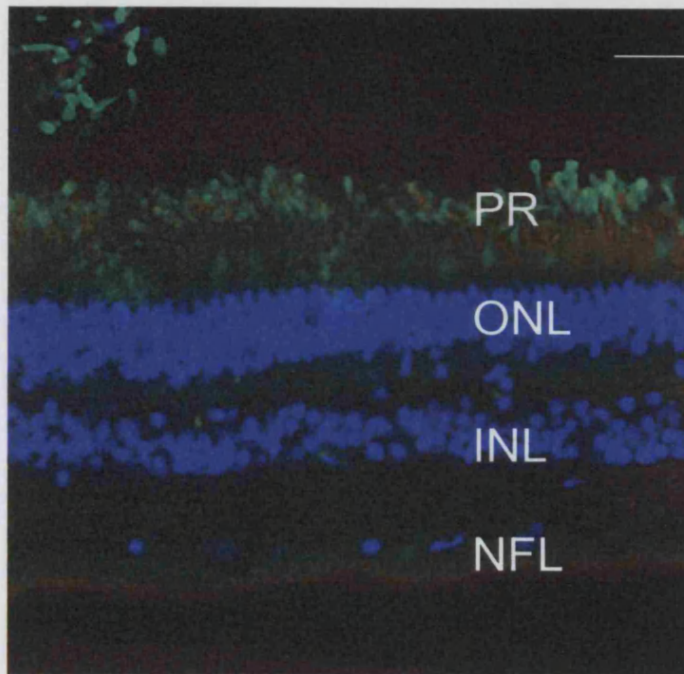


Figure 3.10 Normal human retina immunostaining using antibodies to BDNF (red) and rhodopsin (green). Nuclei are stained with To-Pro3-Iodide (blue).

This image demonstrates weak staining for BDNF at the NFL and the PR layers in normal human retina. There is no staining for BDNF at the nuclear layers.

PR=photoreceptors, ONL=outer nuclear layer, INL=inner nuclear layer, NFL=Nerve fibre layer. Scale bar= 100µm.

Figure 3.11 Double-label immunofluorescence using antibodies to NT4 (red) and rhodopsin (green).

Panel A shows staining for NT4 (red) mainly in the nerve fibre layer (NFL) and photoreceptor (PR) layer. Panel B shows staining for rhodopsin and panel C is the merged image of panels A and B demonstrating a degree of colocalization of NT4 with rhodopsin. PR=photoreceptors, NFL=nerve fibre layer. Scale bar=100µm.

3.8 Immunolocalisation of NT4 in the normal human retina

To determine the expression and distribution of NT4 in the normal human retina double staining of donor sections were examined with confocal scanning laser microscope. This showed positive staining for NT4 mainly in the nerve fibre and photoreceptor layers (figure 3.11). The level of staining was generally low in the sections examined but stronger than those observed in sections stained for BDNF.

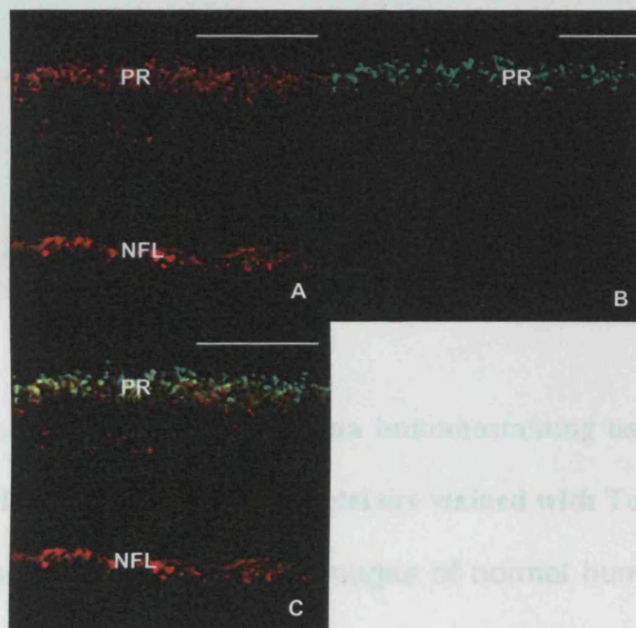


Figure 3.11 Double-labeled immunohistochemistry using antibodies to NT4 (red) and rhodopsin (green).

Panel A shows staining for NT4 (red) mainly in the nerve fibre layer (NFL) and photoreceptor (PR) layer. Panel B shows staining for rhodopsin and panel C is the merged image of panels A and B demonstrating a degree of co-localisation of NT4 with rhodopsin. PR=photoreceptors, NFL=nerve fibre layer. Scale bars=100 μ m.

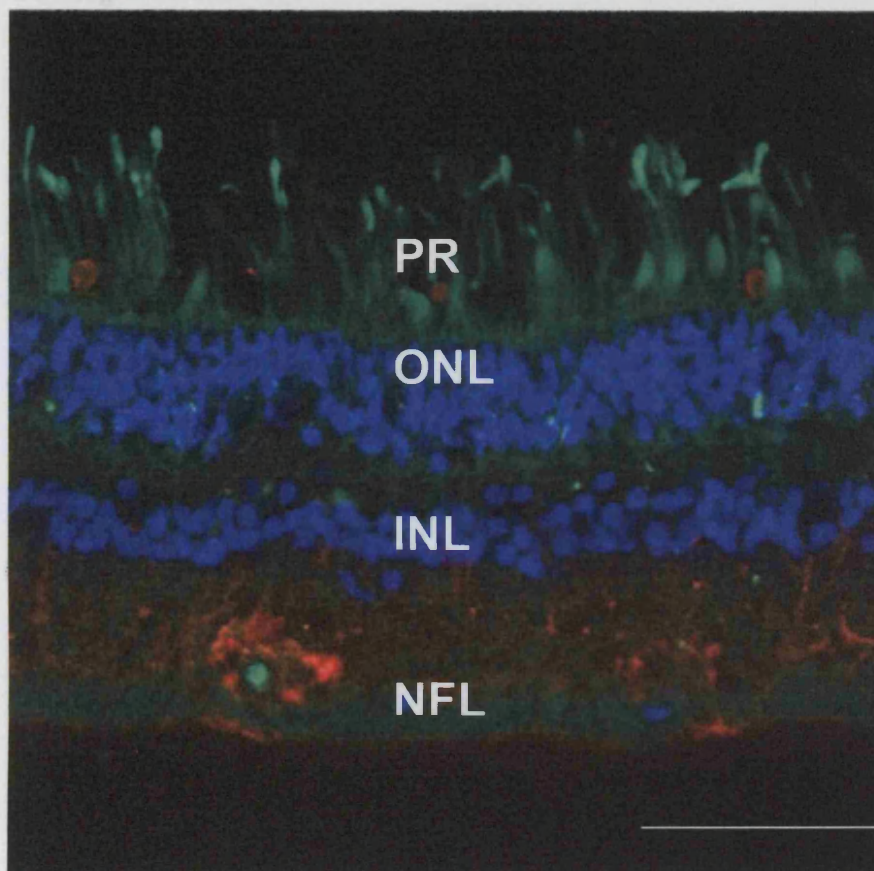


Figure 3.12 Normal human retina immunostaining using antibodies to NT4 (red) and rhodopsin (green). Nuclei are stained with To-Pro3-Iodide (blue).

This scanning confocal laser images of normal human retina shows that NT4 is mainly expressed in the nerve fibre layer and there is no nuclear staining associated with this protein. PR=photoreceptors, ONL=outer nuclear layer, INL=inner nuclear layer, NFL=Nerve fibre layer. Scale bar= 100µm.

3.9 Negative controls

Negative controls used for immunostaining studies including normal human retina sections and PVR retinectomy sections did not show any staining apart from the expected nuclear staining (figure 3.13). Rabbit IgG (DAKO, Golstrup, Denmark) and Mouse IgG (Sigma, St. Lois, MO, USA) were used at the same concentration as the primary antibodies for negative controls. A second negative control was also prepared without addition of primary antibodies.

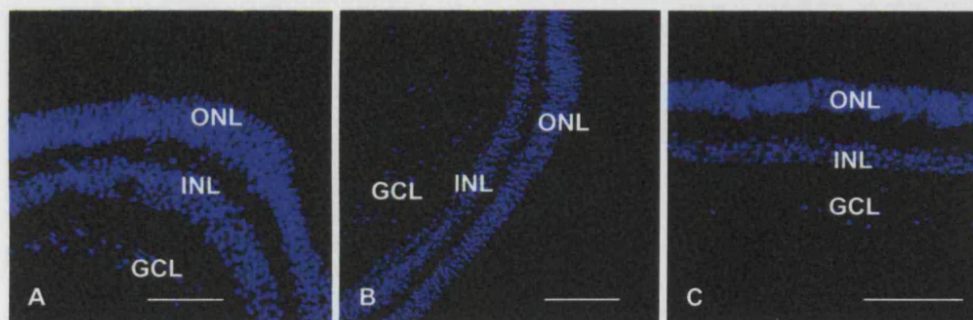


Figure 3.13 Negative control specimens used in immunostaining studies.

This figure shows two of the PVR retinectomy sections (A and B, sample 2, table 3.2) and one of the normal human sections (C) with staining of the nuclear layers of the retina with To-Pro3-iodide in blue. There is no other staining observed in these specimens. ONL= Outer nuclear layer, INL= Inner nuclear layer, GCL= Ganglion cell layer. Scale bar= 100 μ m.

3.10 BDNF expression in PVR retinectomy sections

Eight retinectomy sections from patients with PVR were analysed to investigate any change in degree and pattern of expression of BDNF.

An increased distribution of staining for BDNF throughout the entire retinal section was detected but the intensity of staining was similar to the normal retinal sections studied (figures 3.14 and 3.15). There was no staining for BDNF at the nuclear layers. Photoreceptor outer segments were found to be much less prominent in the PVR retinectomy sections (figures 3.14 and 3.15).

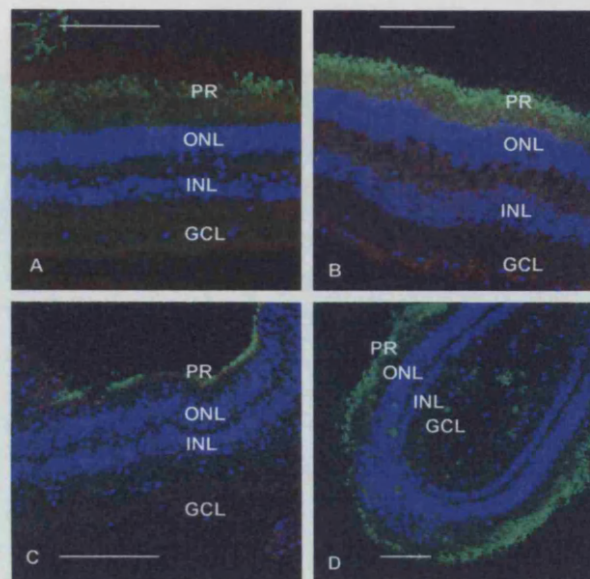


Figure 3.14 Normal (A and B) and PVR retinectomy (C and D) sections immuno-labelled using antibodies to BDNF (red) and rhodopsin (green). Nuclear staining is with To-Pro3-Iodide (blue).

Panels A and B show the pattern of staining in the normal human tissues for comparison. Panels C (sample 1, table 3.2) and D (sample 2, table 3.2) are PVR retinectomy tissues demonstrating a disrupted retinal structure due to PVR. It also shows prominent reduction in the photoreceptor layer particularly in panel C. The intensity and pattern of staining for BDNF (red) in PVR sections was very similar to normal specimens. ONL= Outer nuclear layer, INL= Inner nuclear layer, GCL= Ganglion cell layer. Scale bar= 100µm.

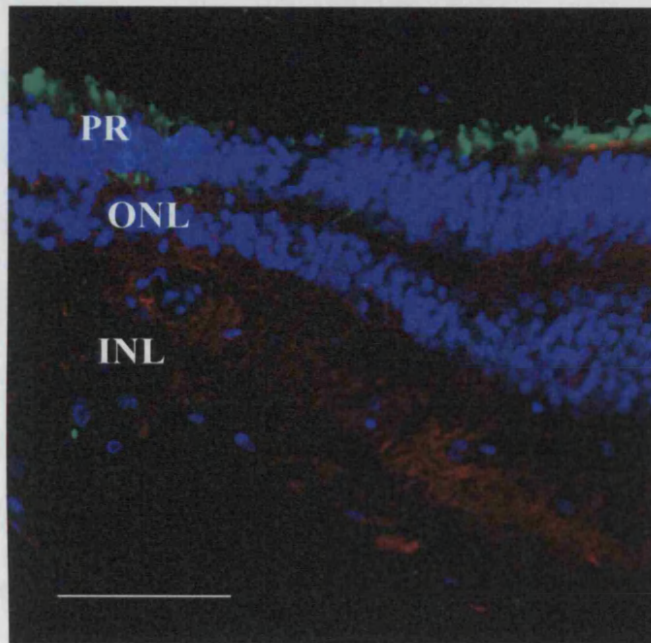


Figure 3.15 PVR retinectomy section double-labeled using antibodies to BDNF (red) and rhodopsin (green). Nuclear staining is with To-Pro3-Iodide (blue).

This section (sample 4, table 3.2) shows a more widespread staining for BDNF (red) in the PVR retinectomy specimen compared to the normal retina shown in figure 3.10. The intensity of staining remained similar to donor retina and only the extent of BDNF distribution seem to have been altered extending throughout the whole thickness of the retina. PR=photoreceptor layer, ONL= Outer nuclear layer, INL= Inner nuclear layer. Scale bar=100µm.

3.11 NT4 expression in PVR retinectomy sections

To determine the level of expression and distribution of NT4 in PVR 8 retinectomy specimens were examined. These sections showed a marked increase in both the intensity and the distribution of NT4 extending the entire thickness of the retina (figures 3.16 A-C). NT4 distribution at the outer retina was however observed to be abruptly limited to where photoreceptor outer segment staining started (figure 3.17). This level corresponds to the outer limiting membrane (OLM), the outer most limit of distribution of the Müller cells.

This pattern of staining and the morphology of the cells stained with NT4 are very suggestive of the presence of this protein in the Müller cells (figure 3.18 and 3.19).

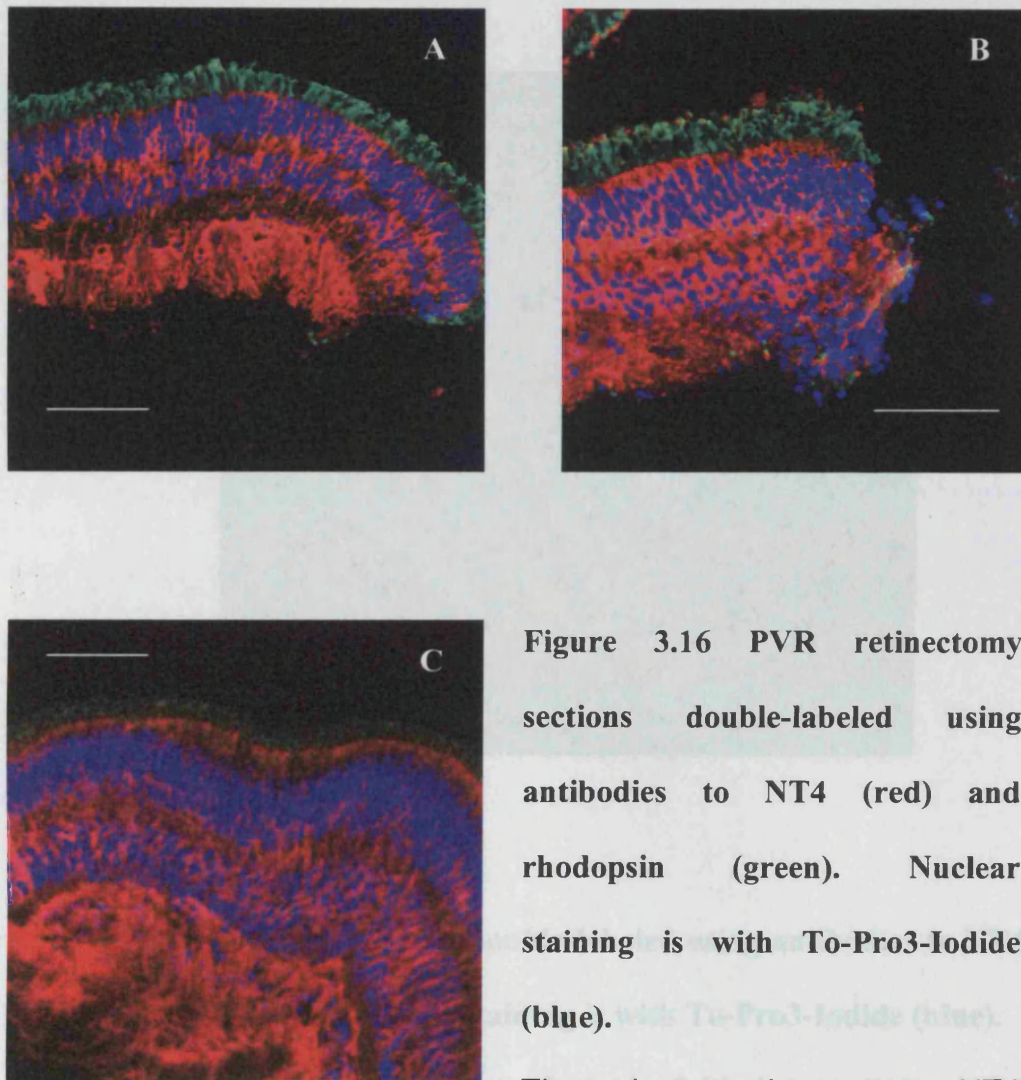


Figure 3.16 PVR retinectomy sections double-labeled using antibodies to NT4 (red) and rhodopsin (green). Nuclear staining is with To-Pro3-Iodide (blue).

These images demonstrate NT4 staining (red) in three different PVR retinectomy sections (panel A=sample 3, panel B=sample 4, and panel C=sample 7 in table 3.2). Compared to the normal retina shown in figure 3.12, there is extensive and widespread expression of NT4 through the whole thickness of the retina with high intensity staining. Scale bar= 100µm.

Limit of staining
at OLM

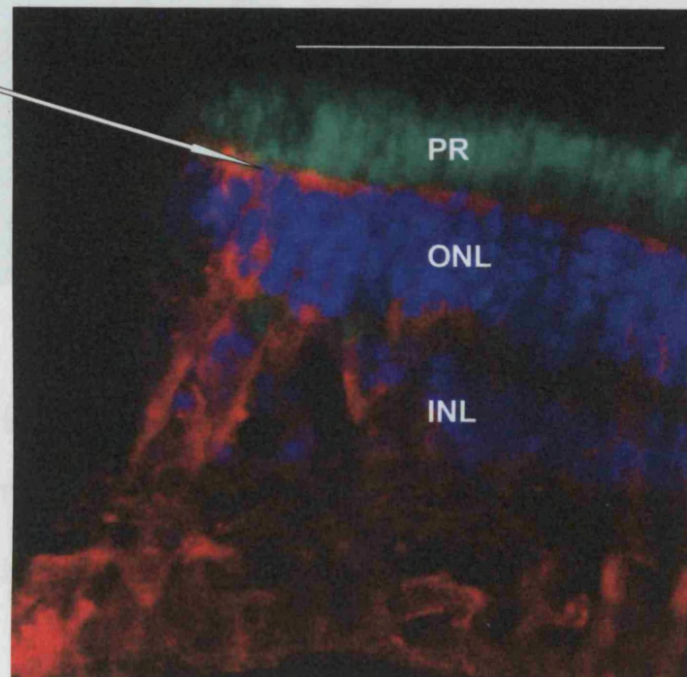


Figure 3.17 PVR retinectomy section double-labeled using antibodies to NT4 (red) and rhodopsin (green). Nuclear staining is with To-Pro3-Iodide (blue).

This PVR retinectomy section (sample 5, table 3.2) demonstrates NT4 staining (red) which is abruptly limited at the same level where photoreceptors outer segment staining is detected (green). This location corresponds to the outer limiting membrane (OLM) of the retina where Müller cell processes end in the outer aspect of the retina. This pattern of staining would also suggest an intercellular location for NT4 as the staining is not spread in the extracellular space. Scale bar= 100 μ m.

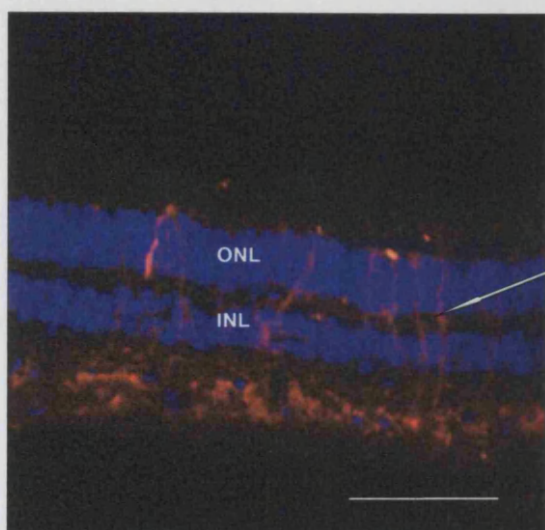


Figure 3.18 PVR retinectomy section double-labeled using antibodies to NT4 (red). Nuclear staining is with To-Pro3-Iodide (blue). This PVR retinectomy specimen (sample 6,

table 3.2) shows NT4 staining (red) distributed through the entire thickness of the retina (arrow) corresponding to that expected from Müller cells distribution. The morphology of the cells stained is also similar to Müller cells. Scale bar= 100 μ m.

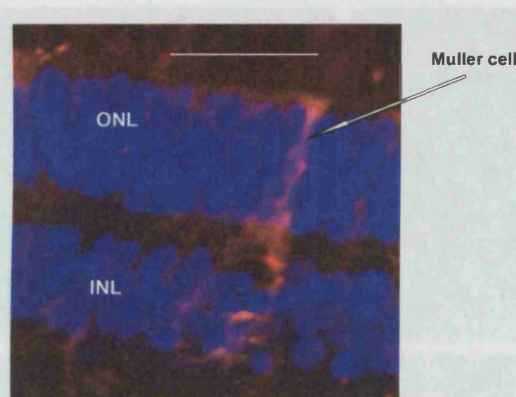


Figure 3.19 Magnified view of figure 3.18.

This image shows cell morphology of remarkable similarity to a single Müller cell with its characteristic cell processes and spanning across inner and outer nuclear layers. ONL= outer nuclear layer, INL=inner nuclear layer. Scale bar= 50 μ m.

3.12 NT4 staining in PVR co-localises to Müller cells

To further investigate the location of NT4 staining in the PVR specimens we co-stained the sections with both GFAP and NT4. GFAP is generally up-regulated in the retina in PVR and is a known marker for retinal glial cells. In the studied sections there was GFAP staining extending the whole thickness of the retina as would be expected in PVR (figures 3.20 and 3.21). This experiment confirmed co-localisation of GFAP with NT4 providing evidence that up-regulated NT4, observed in PVR sections studied, is contained within Müller cells.

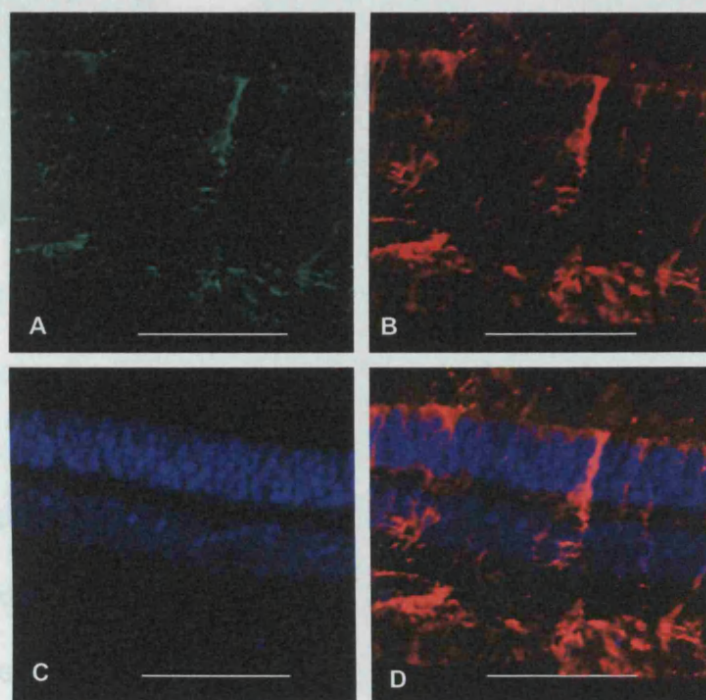


Figure 3.20 PVR retinectomy sections double-labeled using antibodies to NT4 (red) and GFAP (green). Nuclear staining is with To-Pro3-Iodide (blue).

The morphology of cells staining for NT4 and their co-localisation with GFAP is highly suggestive of NT4's presence within Müller cells. Scale bars=100µm.

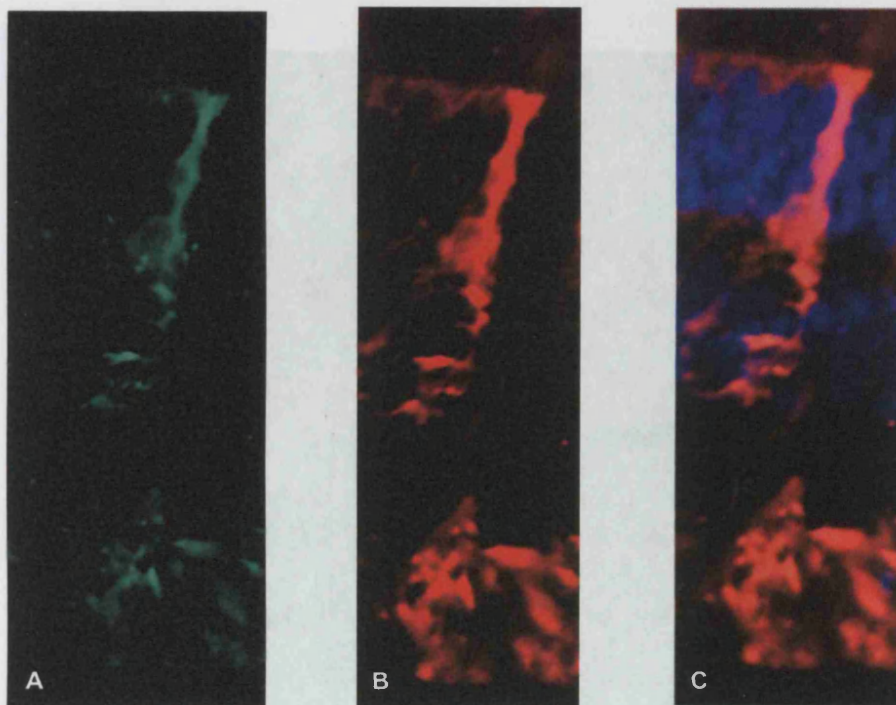


Figure 3.21 Magnified view of figure 3.20.

These images show a section from an eye with PVR co-staining for GFAP (red) and anti rhodopsin antibody (green). Nuclear staining is with To-Pro3-Iodide (blue). Panel A, green stain) and NT4 (panel B, red stain). Panel C shows nuclear staining (blue) to demonstrate the retinal architecture. It shows very good co-localisation of NT4 and GFAP (panel C) providing evidence for intracellular distribution of NT4 in Müller cells.

PR=photoreceptor layer, ONL=outer nuclear layer, INL=inner nuclear layer, NFL=nerve fibre layer. Scale bar= 100µm.

3.13 TrkB expression in normal human retina

TrkB receptor expression in the normal human retina was examined by double staining the sections with the antibody for the full length receptor.

There was minimal staining at the nerve fibre and photoreceptor layers in the studied sections. No staining was observed at the nuclear layers.

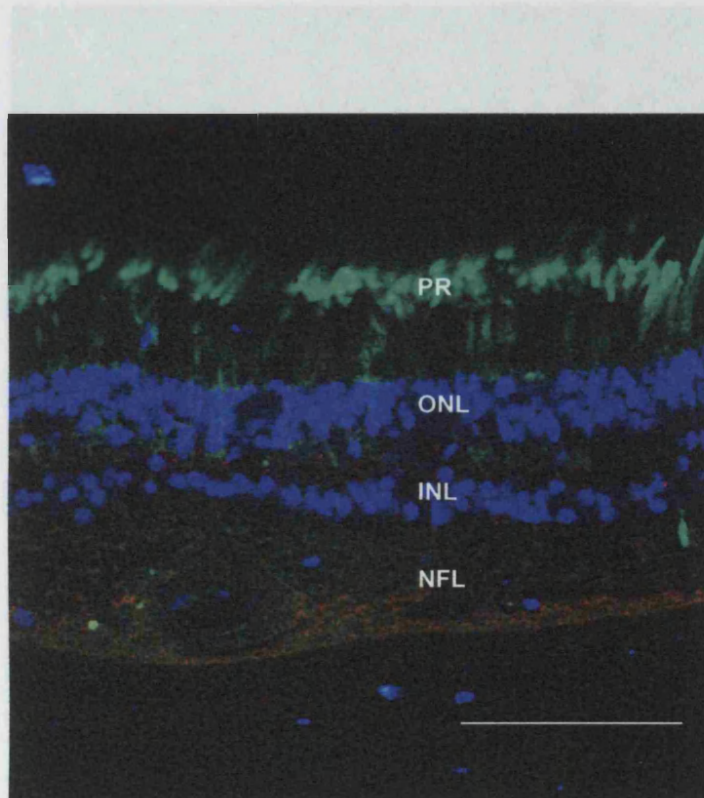


Figure 3.22 Normal human retina immunostained using anti TrkB antibody (red) and anti rhodopsin antibody (green). Nuclear staining is with To-Pro3-Iodide (blue).

These images show minimal staining for TrkB (red) receptor detectable at the ganglion cell layer. There is no staining at the nuclear layers. This PVR specimen (see table 3.2) shows no staining for the receptor. Photoreceptor receptor staining is present (green) suggesting that technical problems with staining are unlikely. Scale bar= 100µm.

3.14 TrkB expression in PVR retinectomy sections

PVR retinectomy sections were examined to assess pattern and level of expression of TrkB compared to the normal retina. No staining was detectable in any of the sections studied. There were also no detectable truncated receptors using this method of staining.

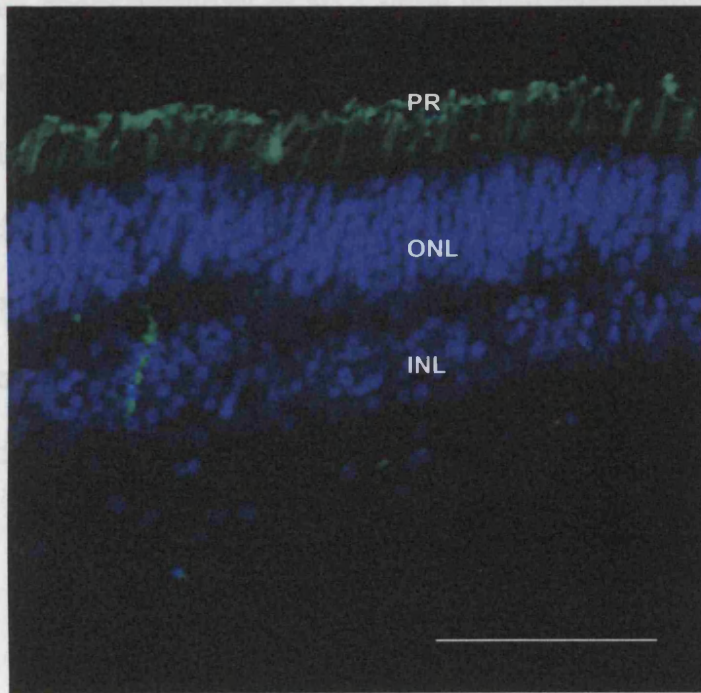


Figure 3.23 PVR retinectomy section immunostained using anti TrkB antibody (red, no staining here) and anti rhodopsin antibody (green). Nuclear staining is with To-Pro3-Iodide (blue).

This PVR specimen (sample 3, table 3.2) shows no staining for the receptor. Photoreceptor receptor staining is present (green) suggesting that technical problems with staining are unlikely. Scale bar= 100µm.

3.15 t-TrkB mRNA levels in cultured MIO-M1 cells supplemented with NT4

We next studied the possible effect of increased NT4 levels on the expression of t-TrkB mRNA. Cultured MIO-M1 cells supplemented with various concentrations of rhNT4 for 5 days were assayed using RT-PCR. There were detectable bands corresponding to the expected size for t-TrkB for all concentrations of NT4 used in the experiments. The strongest band was detected with NT4 concentration of 10ng/ml and the weakest intensity band was seen at the concentration of 1ng/ml. When 50ng/ml of NT4 used the band intensity produced an intermediate signal (figure 3.24). It appears that the lower (1ng/ml) and higher (50ng/ml) concentrations of NT4 used in this experiment may have the same effect on the level of t-TrkB production and reduce its mRNA production.

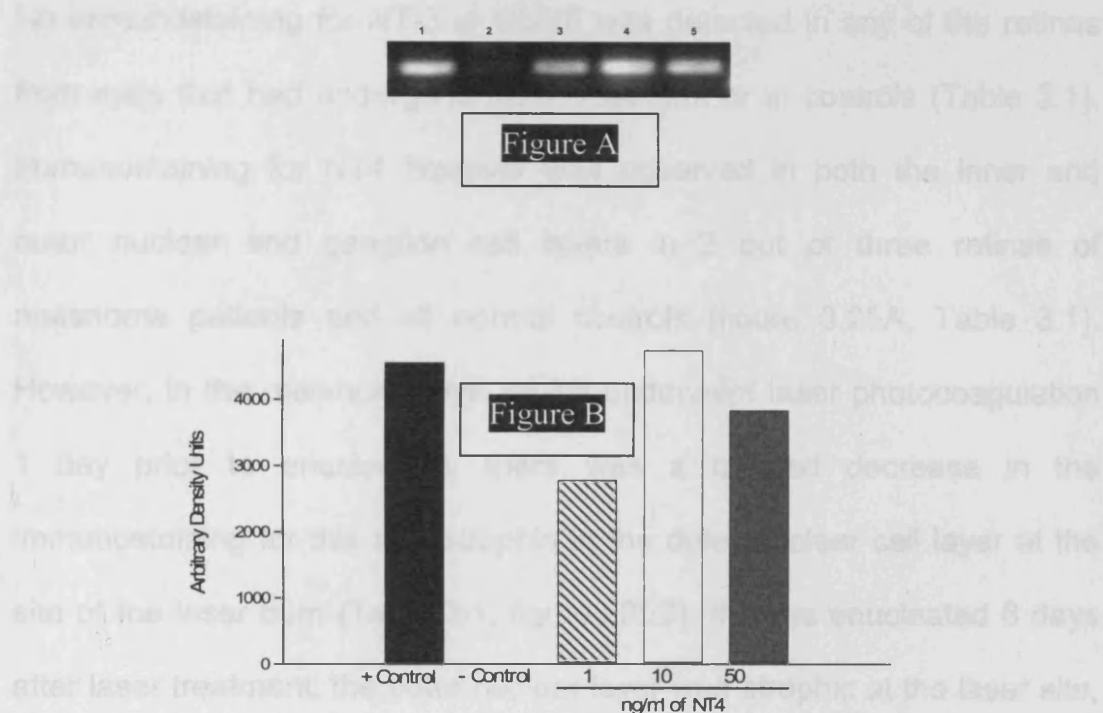


Figure 3.24 t-TrkB mRNA levels in cultured Müller cells with NT4 supplement (figure A). Density measurements for RT-PCR products (figure B).

RNA was extracted after 5 days of cell culture. The band intensity in lane 4 (sample treated with 10ng/ml NT4) was strongest. Both lower (lane 3) and higher (lane 5) concentrations of the NT4 supplement to the culture medium resulted in detection of lower intensity bands compared to lane 4. Lane orders; 1= control with no NT4 added to the culture medium, 2= negative control, 3= 1ng/ml NT4 added to the culture medium, 4= 10ng/ml NT4 added to the culture medium, 5= 50ng/ml NT4 added to the culture medium (figure A). Intensity of the RT-PCR bands was quantified in arbitrary units using the Kodak computer software programme KDS1D – version 2.0 (figure B).

3.16 Neurotrophins expression in retinas of patients with choroidal melanoma and in controls

No immunostaining for NT-3 or BDNF was detected in any of the retinas from eyes that had undergone laser treatment or in controls (Table 3.1). Immunostaining for NT4 however was observed in both the inner and outer nuclear and ganglion cell layers in 2 out of three retinas of melanoma patients and all normal controls (figure 3.25A, Table 3.1). However, in the melanoma eye, which underwent laser photocoagulation 1 day prior to enucleation, there was a marked decrease in the immunostaining for this neurotrophin in the outer nuclear cell layer at the site of the laser burn (Table 3.1, figure3.25B). In eyes enucleated 6 days after laser treatment, the outer nuclear layer was atrophic at the laser site, but occasional cells still present, were negative for this molecule (figure3.25C).

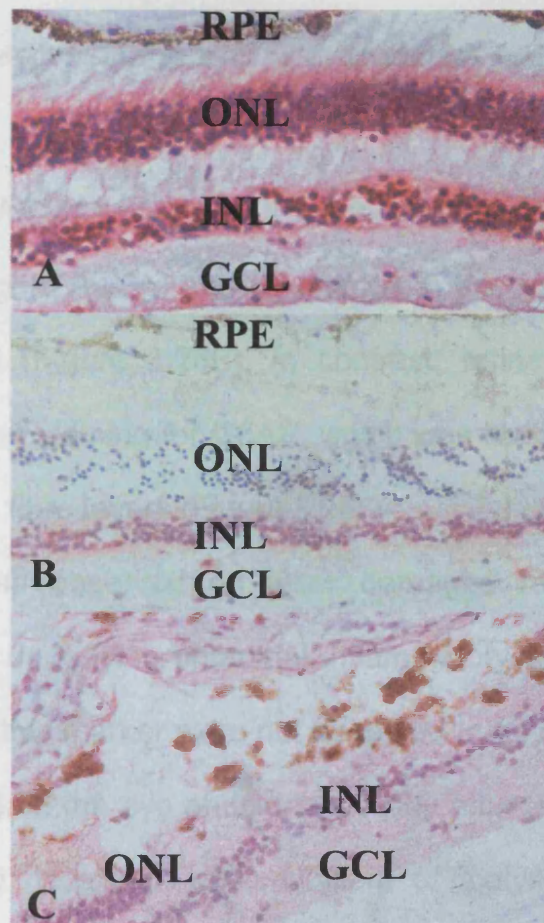


Figure 3.25: Pattern of NT4 staining in normal and laser treated retinas H&E and anti-NT4 stain (from Ghazi-Nouri SM *et al* 2003).

FigA: Normal retina showing intense NT4 staining in inner and outer nuclear layers, 50X magnifications. FigB: one day post laser showing reduced NT4 staining in ONL. This reduced staining is confined to the extent of laser injury, 50 X magnifications. Fig C: six days post laser showing atrophic ONL at the site of laser with only a few cells seen and reduced NT4 staining, 100X magnification. RPE=retinal pigment epithelium, ONL=outer nuclear layer, INL=inner nuclear layer, GCL=ganglion cell layer.

3.17 GFAP and CD68 expression in eyes with choroidal melanoma and in controls

Intense staining for GFAP was observed throughout all the retinal layers in all melanoma eyes (Figure 3.26A) and no change in this expression was found in lasered eyes (Figure 3.26B). In contrast, retina from normal controls showed minimal staining for GFAP, which was limited to the ILM, ganglion cell and nerve fibre layers (Figure 3.26C).

Staining for CD68 was observed on laser damaged retinal pigment epithelial cells and on underlying choroidal inflammatory and pigmented cells. Cells positive for this marker were not observed in any of the control specimens (Fig. 3.27A). In the eye enucleated 1 day after laser treatment there was a marked localized accumulation of polymorphonuclear leucocytes, mononuclear cells and pigmented choroidal cells at the laser site (Figs. 3.27B). In addition, a moderate number of scattered cells staining for CD68 were observed in the choroid, and mild RPE cells staining for CD68 was seen (Fig 3.27B). In the eye enucleated 2 days after laser, a more intense infiltration of CD68 positive cells, some of which formed an incomplete perivascular cuff, was observed, and RPE cells displayed a more intense CD68 staining than on day 1 (Figure 3.27C). In contrast, intense staining for CD68 was observed on mononuclear and choroidal pigmented cells as well as RPE cells 6 days after laser treatment (Figs 3.27D).

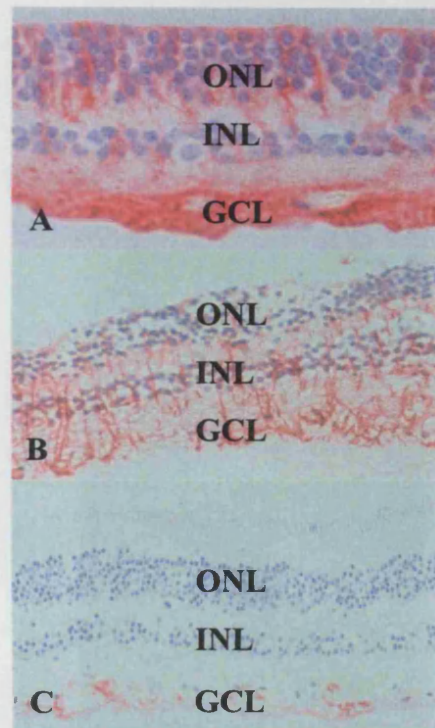


Figure 3.26 Pattern of GFAP staining in malignant melanoma, laser treated and normal Retinas. H&E and anti-GFAP stain (red) (from Ghazi-Nouri SM *et al* 2003).

Fig.A: GFAP staining throughout all layers of retina in malignant melanoma, 50X magnifications. Fig.B: GFAP staining throughout all layers of the lasered retina (1 day post laser), 50 X magnifications. Fig.C: GFAP staining of the inner limiting membrane, Ganglion cell and nerve fiber layer in normal retina, 50 X magnifications. ONL=outer nuclear layer, INL=inner nuclear layer, GCL=ganglion cell layer.

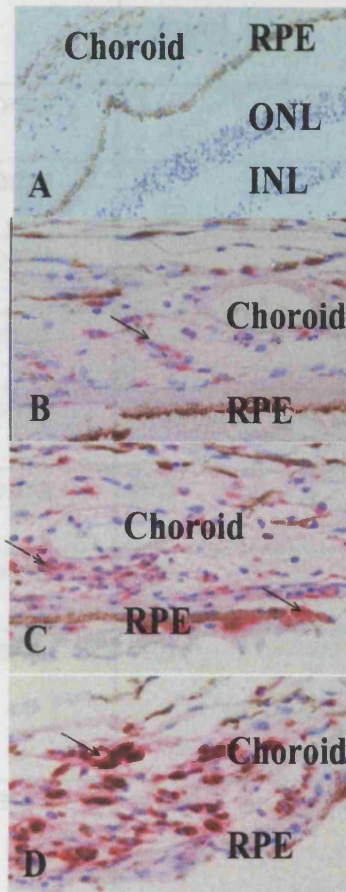


Figure 3.27 Pattern of CD68 staining in normal and laser treated retinas. H&E and anti-CD68 antibody (from Ghazi-Nouri SM *et al* 2003).

Fig.A: Normal retina with no CD 68 staining in the choroid, 50X magnifications. Fig.B: CD68 staining in the choroid 1 day post laser, 100 X magnifications. Fig.C: CD68 staining 2 days post laser. Note CD68 positive cells associated with RPE and perivascular cuff, 100 X magnifications. Fig.D: CD68 staining 6 days post laser showing more intense staining compared to days one and two, 100X magnification. RPE=retinal pigment epithelium, ONL=outer nuclear layer, INL=inner nuclear layer.

Specimen number	Origin of specimen	Laser prior to enucleation (days)	NT4	GFAP	CD68
1	Melanoma	1	+ ^a	+++ ^c	+
2	Melanoma	2	+ ^a	+++ ^c	+
3	Melanoma	6	+ ^a	+++ ^c	++
4	Melanoma	6	+ ^a	+++ ^c	++
5	Melanoma	n/a	+ ^b	+++ ^c	–
6	Melanoma	n/a	+ ^b	+++ ^c	–
7	Melanoma	n/a	–	+++ ^c	–
8	Cadaveric donor	n/a	++ ^b	+ ^d	–
9	Cadaveric donor	n/a	++ ^b	+ ^d	–
10	Cadaveric donor	n/a	++ ^b	+ ^d	–

Table 3.1 Immunohistochemical staining of lasered and control retinas

Intensity of staining: +++ = strong, ++ = moderate, + = mild, – = negative

^a Staining reduced in outer nuclear layer at the site of the laser burn, ^b staining of inner and outer nuclear layers of the retina, ^c staining throughout the whole retina, ^d scattered staining of cells predominantly in the nerve fibre layer.

Sample (age,sex)	Condition	BDNF (staining distribution)	NT4 (staining distribution)	TrkB (staining distribution)	t-TrkB (staining distribution)
1 (80,F)	PVR	+ (ILM to OLM)	+++ (ILM to OLM)	-	-
2 (56,F)	PVR	+ (ILM to OLM)	++ (ILM to OLM)	-	-
3 (72,F)	PVR	+ (ILM to OLM)	+++ (ILM to OLM)	-	-
4 (67,F)	PVR	+ (ILM to OLM)	+++ (ILM to OLM)	-	-
5 (70,M)	PVR	Sample lost during processing	+++ (ILM to OLM)	-	-
6 (52,M)	PVR	+ (PR)	+++ (ILM to OLM)	-	-
7 (55,M)	PVR	+ (NFL and PR)	++ (ILM to OLM)	-	-
8 (38,M)	PVR	+ (ILM to OLM)	+++ (ILM to OLM)	-	-
9 (55,M)	Cadaveric donor	+ (NFL and PR)	+ (NFL and PR)	+ (NFL and PR)	+ (NFL and PR)
10 (50,M)	Cadaveric donor	+ (NFL and PR)	+ (NFL and PR)	+ (NFL and PR)	+ (NFL and PR)
11 (75,M)	Cadaveric donor	+ (NFL and PR)	+ (NFL and PR)	+ (NFL and PR)	+ (NFL and PR)

Table 3.2 Immunostaining intensity and distribution for BDNF, NT4, TrkB and t-TrkB observed in PVR retinectomy sections and normal controls.

ILM=inner limiting membrane, OLM=outer limiting membrane, NFL=nerve fibre layer, PR=photoreceptors. Intensity of staining: +++ = strong, ++ = moderate, + = mild, - = negative.

Sample (age,sex)	Aetiology of RD	Silicone oil tamponade	Number of VR procedures prior to sample analysis	Time between first RD surgery and retinectomy specimen (months)	Comments
1 (80,F)	RRD	Yes	2	17	APVR, SRM
2 (56,F)	RRD	Yes	4	1	PVR, ERM
3 (72,F)	RRD (inferior RD)	Yes	3	3	PVR, Total RD
4 (67,F)	RRD	No	2	2	APVR
5 (70,M)	RRD	Yes	3	12	PVR, Inferior RD
6 (52,M)	RRD	Yes	4	2	PVR
7 (55,M)	RRD	Yes	4	3	PVR, Inferior TRD, ERM
8 (38,M)	Penetrating injury	Yes	3	27	PVR, Subtotal RD, SRM

Table 3.3 Details of patients and retinectomy tissues studied.

RD=retinal detachment, RRD=rhegmatogenous RD, TRD=tractional RD,

PVR= proliferative vitreoretinopathy, APVR= anterior PVR,

SRM=subretinal membrane, ERM=epiretinal membrane.

Chapter 4

Discussion

Exogenous BDNF is known to rescue ganglion cell death after optic nerve injury (Mansour-Robaye S 1994). Its mechanism of action is believed to be indirect via glial cells in the retina. Müller cells are the principal glial cells of the human retina and span its entire thickness, being in direct contact with every type of neuron in the retina. Wahlin KJ *et al* (2000) data suggest that BDNF, CNTF, and FGF2 may exert their effects on photoreceptors by acting indirectly through activation of Müller cells and perhaps other nonphotoreceptor cells. Although there are reports in the literature suggesting the existence of NTs in various parts of the eye including the lamina cribrosa (Lambert W 2001) and the trabecular meshwork (Wordinger RJ 2000), the exact location of NT production in the retina has not been fully characterised.

The findings presented here confirm the only previous report (Oku H 2002) that cultured human Müller cells express BDNF at mRNA level. In their study, Oku *et al* investigated primary cultured Müller cells whereas the present study examined the expression of this factor by a Müller cell line. Oku *et al* also observed that cultured Müller cells expressed NGF and NT3 but they did not investigate the expression of NT4, and there is no other report in the literature indicating the expression of NT4 by human Müller cells. The present results however demonstrate that cultured MIO-M1 cells express NT4 at mRNA level. The expression of NT4 as judged by RT-PCR experiments was much lower than that of BDNF. This was inferred from the observation that bands corresponding to the NT4 products were less intense than those of the BDNF products resulting from the amplification of equal amounts of mRNA. Quantitative PCR

techniques were not used here to demonstrate this difference accurately, nevertheless, control amplification of the house keeping gene GAPDH showed similar levels of mRNA in the samples investigated, for which differences in the intensity of the bands observed strongly suggests that the MIO-M1 levels of mRNA expression coding for NT4 were lower than those coding for BDNF.

It was of interest that although mRNA coding for NT4 was detected in the cells, protein expression for this molecule could not be identified by Western blot analysis of cultured MIO-M1 cell lysates or supernatants. It is possible that the amount of protein produced was below detectable levels using the methods described in this study. This may be explained by the low mRNA levels of NT4 detected in the RT-PCR experiments. Other possible explanation is that translation of mRNA into protein did not occur under the conditions used in the study. However, this is unlikely since immunostaining for the NT4 protein in fixed Müller cells demonstrated positive staining for this molecule (see below) and Western blot analysis of the control protein yielded a band corresponding to the molecular weight for NT4 (14 kD).

Anti-BDNF antibodies detected a band of approximately 40 kDa in Western blot experiments. This is not the expected band for the monomer of this protein and probably represents the pro-protein which has been reported in other studies (Seidah NG 1996, Fayard B 2005). All NTs are generated from 31-35 kDa precursors, which are then cleaved intracellularly to produce the mature molecule. Although the band detected in the present study appears to be of a slightly larger size, the fact that a

band of the same molecular weight was detected in the appropriate positive control (mouse brain extract) suggests that the antibody had positively identified BDNF protein. Failure to detect BDNF at its monomeric size may be due to its absence in the MIO-M1 cell lysates or it may be that there was very small amount of this protein present and the abundance of the pro-protein had led to only one identifiable band.

BDNF is reported to protect ganglion cells from ischemic stress (Unoki K, and LaVail MM 1994) and after an optic nerve injury (Peinado-Ramon P 1996, Mansour-Robaye S 1994). There is also evidence suggesting that BDNF and NGF can rescue photoreceptor cells in animal models of retinal detachment (Lewis GP 1999) and retinal light damage (Harada T 2000), respectively. Expression of this neurotrophic factor by Müller cells suggests a neuroprotective role for these cells. The amount of BDNF production can also be altered in inflammatory conditions. This was demonstrated in this study when PDGF supplement was added to the culture medium. This resulted in an increased expression of mRNA coding for BDNF, suggesting that neurotrophic factors expression may be induced by this factor, which is highly upregulated during retinal inflammatory processes. PDGF is released from aggregated platelets at wound sites and acts as a chemo-attractant and mitogen for many cell types that participate in wound repair, including monocytes, macrophages, and fibroblasts (Pierce GF 1991). Exogenous PDGF promotes increased wound strength (Pierce GF 1989 and 1991) and is also known to play an important role in proliferative retinopathies (Smith-Thomas L 1996). It could therefore be expected that elevated expression of BDNF and other

NTs may be found in proliferative vitreoretinopathy. There are no previous studies demonstrating that these factors are increased during the development of this condition, and the present study shows that both BDNF and NT4 expression is highly increased in human PVR tissues as shown by immunohistochemistry and scanning confocal laser images (further discussed below).

Immunostaining of fixed Müller cells demonstrated intracytoplasmic presence of BDNF and NT4 in a similar pattern of distribution; mainly granular perinuclear with some positive staining in the cell processes. Presence of trophic factors within Müller cells further confirms suggestions that these cells exert play an important role in neural support. It has already been suggested that the trophic effects of NTs on photoreceptors may be exerted indirectly via Müller cells (Wahlin KJ 2000). It has been postulated that these trophic factors are transported throughout the whole thickness of the retina within the Müller cells (von Bartheld 2001), and that NTs may be internalised by binding to the truncated receptors (Eide FF 1996). Internalised NTs are not necessarily degraded and can be targeted for release or for packaging in vesicles for anterograde axonal transport after internalisation (von Bartheld 2001). Müller cells, spanning the entire thickness of the retina have structural advantage to achieve this task.

This study demonstrated expression of the truncated isoform of the TrkB receptor by MIO-M1 cells both at the mRNA and protein levels. The role of truncated TrkB is believed to be the internalisation of its ligands and to function as an inhibitory modulator of neurotrophin responsiveness (Eide FF 1996). However, this study's results did not show expression of the full-

length receptor by these cells. This contrasts with that reported by Oku *et al* (2002) who showed mRNA expression of the full-length receptor (TrkB and TrkC) by cultured human Müller cells. Oku *et al* did not use a cell line and it is possible that cell line used in the present study does not express the full-length receptor. Co-expression of the truncated TrkB and both BDNF and NT-4 found in this study also suggests an auto-regulatory function in these cells. In relation to the detection of TrkB, this study has some limitations, including lack of a suitable positive control for TrkB in RT-PCR and Western blot analysis. However, controlled amplification of GAPDH yielded a band with the expected size of 600bp and the RT-PCR product exhibited the molecular weight of the expected size. This however suggests that TrkB detection was specific, and it is supported by the Western blot analysis of this receptor, which yielded bands of molecular weight corresponding to t-TrkB.

There is extensive non-neuronal localization of truncated TrkB-T1 receptors in the central nervous system (Fryer RH 1997). There is also an up-regulation of the truncated receptors by CNS glial cells in response to injury leading to the speculation that these receptors may sequester BDNF and NT-4/5 to reduce their local availability and, thus, limit axonal sprouting (Fryer RH 1997). Findings from other investigators, however, suggested that truncated TrkB-T1 mediated neurotrophic-evoked calcium signalling in glial cells (Rose CR 2003).

Although interesting observations with respect to the expression of various NTs and its receptors by the Müller glial cell line MIO-M1 were observed in this study, it might be possible that differences between these cells and

freshly isolated cells from the retina may exist. Further investigations are needed to clarify this issue.

In this study the expression of receptors in the retinectomy specimens was not detected, unlike the retinal specimens from normal donor eyes, which show a mild expression of this molecule. The intracellular pattern of distribution of TrkB in cultured Müller cells is consistent with previous reports that in some projection neurons from the central nervous system, Trk receptors appear to be largely sequestered in intracellular vesicles (Meyer-Franke A 1998). The same study also suggested that only in the presence of a second signal, such as cAMP or Ca^{2+} , the receptors are inserted efficiently into the plasmalemma.

Down-regulation of TrkB may be a direct result of the well recognised phenomenon whereby excessive presence of ligand leads to consumption of the available receptor and also its reduced synthesis. Results in the present study suggest that varying the concentrations of NT4 can influence the amount of the t-TrkB mRNA synthesis. Addition of human NT4 to MIO-M1 cells cultured medium resulted in changes to the level of t-TrkB mRNA detected by RT-PCR. Addition of human NT4 at concentrations of 1ng/ml and 50ng/ml to cultured MIO-M1 cells resulted in a reduced t-TrkB mRNA production compared with that observed when 10ng/ml of the NT4 supplement was added. It is not clear what level of growth factor may be present under pathologic conditions and this experiment merely demonstrates that NT4 concentration can influence mRNA expression of t-TrkB suggesting a negative regulatory effect of NT4 on TrkB receptors. This observation is supported by previous reports that

show a down regulation of full length TrkB in response to BDNF exposure both in *vitro* and *in vivo* (Frank I 1996, Knusel B 1997). In contrast, Kunsel *et al* only observed down regulation of the full length TrkB receptor and not the truncated isoform.

Reduced receptor expression was associated with increased expression of both BDNF and NT4 in all 8 PVR retinectomy sections investigated in the present study. Staining for BDNF in all tissues examined showed minimal increase with extensive distribution of this protein throughout the retina. In contrast NT4 was markedly up regulated, showing widespread and intense distribution throughout the whole thickness of the retina. This staining abruptly stopped at the level where photoreceptor outer segments started i.e. at the level of the outer limiting membrane. This pattern of distribution suggested that NT4 was most likely expressed by Müller cells. Furthermore the morphology and distribution of stained cells expressing NT4 was very similar to Müller cells. Co-localisation of NT-4 and GFAP confirmed the presence of NT-4 within Müller cells.

Reduced availability of receptors would result in diminished effectiveness of NTs. Where exogenous NT factors are introduced to promote neuronal survival, it is also important to consider whether receptors are synthesised and expressed to utilise their ligands. Cheng *et al* (Cheng L 2002) have shown that the positive short-term effect of endogenous BDNF in ganglion cell survival can be enhanced when the appropriate receptor is also administered at the same time. This finding follows their observation that NT receptors are down regulated following optic nerve injury.

In retinal injury there is hypertrophy of glial cells and enhanced immunoreactivity for GFAP (Bignami A 1979, Osborne NN 1991). This is illustrated by the study of Honjo *et al* (Honjo M 2000), who observed that expression of GFAP and ciliary neurotrophic factor (CNTF) in Müller cells is upregulated following an intravitreal injection of N-methyl-D-aspartate (NMDA). This indicates that Müller cells express neurotrophins in response to NMDA retinal toxicity. This is consistent with the present findings that neurotrophic factor upregulation can be observed following retinal damage.

Immunohistochemical findings on paraffin embedded sections in this study showed that normal human retina expresses NT4 at the level of outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) and that NT4 expression was down-regulated at the site of laser burns when examined at 1, 2 and 6 days post-laser. The degree of down regulation was more marked in the eyes enucleated 6 days after laser treatment and less on those enucleated after 1-2 days. These observations suggest that NT4 down regulation may be either secondary to local cell damage at ONL level or it may reflect a true reduction in NT4 expression in cells normally producing these proteins.

There was a difference in the pattern of distribution of NT4 observed between paraffin embedded and PFA fixed samples; in paraffin embedded control samples NT4 staining is observed in the inner and outer nuclear layers whereas in PFA fixed control samples the staining is more prominent in the nerve fibre and photoreceptor layers. This may be due to one or combination of several factors; Anti NT4 antibodies used in these

two sets of experiments were from two different suppliers and their specificity might be different. Furthermore different techniques were employed to prepare the samples prior to imaging. PFA fixation tends to have better preservation of epitopes whereas paraffin may have deleterious effects by masking some epitopes.

Retinal specimens from melanoma eyes showed reduced intensity of NT4 staining in 2 samples and absent NT4 staining in one case. This suggests reduced NT4 production, increased NT4 degradation or a combination of both. It is also possible that the observed GFAP up-regulation, indicating Müller cells reactivity, in melanoma and laser treated retinas is associated with the reduced NT4 staining. *In vitro* studies suggest that there is an increased sequestration of BDNF and NT4 at the site of reactive gliosis in the central nervous system (Alderson RF 2000, Fryer RH 1997).

Whereas in this study reactive gliosis in PVR retinectomy sections is associated with increased expression of NT4, the opposite is observed in melanoma affected samples with reduced NT4 expression associated with reactive gliosis. These contrasting pictures suggest that glial cell hypertrophy is not always associated with increased expression of neurotrophins and the cause of gliosis and the underlying complex interactions of inflammatory agents may differ in different conditions, leading to contrasting results.

The immunohistochemical staining on paraffin embedded sections used in this study did not detect BDNF or NT3 in any of the retinas examined. These findings do not correlate with previous reports in a variety of vertebrate retinas (Das I 1997, Bennett JL 1999, Hallbook F 1996,

Cellerino A 1997). It may be that the antibodies used in this study did not recognise the epitopes expressed in paraffin tissues, or that the method used was not sensitive enough. Strong expression of GFAP was observed in control melanoma eyes as well as laser treated eyes, but minimal expression of these molecules was seen in normal controls. Increased levels of this protein has been reported in the Müller cells following laser exposure in rabbit eyes (Humphrey MF 1993 and 1997) where GFAP immunoreactivity in Müller cells was observed as far as 4-5 mm from the laser foci which was much larger than the RPE disrupted area (Humphrey MF 1993). Findings presented here suggest that retinal GFAP expression is increased in the presence of melanoma and laser treatment did not modify this expression. It is of interest that an increased immunoreactivity for GFAP has also been observed in retinal explants when exposed to choroidal melanoma cells ex vivo (Enzmann v 2000). GFAP may be maximally expressed in melanoma eyes and therefore it may not be possible to detect a further increase using immunocytochemical techniques following laser treatment.

The expression of CD68 was only observed at the site of the laser treated retinas and not in the normal or melanoma affected eyes. Immunoreactive cells were localised in the laser damaged RPE as well as in the underlying choroid. CD68 is a cell surface marker for macrophages and has previously been reported to be expressed by cultured RPE cells (Limb GA 1997, Elner SG 1992).

Macrophages and leucocyte infiltrates characterise inflammatory responses and their presence in the choroid in foci of laser disruption may

indicate an inflammatory response induced by this treatment. Previous studies have shown that the origin of the mononuclear leukocytes that accumulate at the site of laser burns is the systemic circulation (Martini B 1992, Ishikawa Y 1983).

Findings here agree with those previously reported (Nicolai U 1993) where RPE cells in specimens obtained from eyes with various ocular diseases including intraocular melanoma did not react with antibody to CD68. This observation suggests that laser damaged RPE cells and those cultured (Limb GA 1997, Cellerino A 1997) ("normal") may have undergone activation, not seen in normal cells *in vivo*.

Future directions

Since Müller cells span the entire thickness of the retina, gene delivery to these cells is a useful strategy to provide protection to all retinal cell types. It may be possible to stimulate these cells to provide neuroprotection given that over expression of BDNF was observed in response to PDGF, an important inflammatory factor produced during retinal inflammatory conditions such as PVR. The exact role of NT4 in the human retina is yet to be determined but it would be of importance to investigate the effect of various factors on NT4 production by Müller cells, as well as to determine the possible autocrine role of Müller cells given the coexpression of BDNF, NT4 and their common receptor TrkB.

Vascular endothelial growth factor (VEGF) expression by Müller cells is also of particular interest due to the important role of this angiogenic factor in normal retinal vasculature development and in pathological conditions such as age related macular degeneration and diabetic retinopathy. Since BDNF has been shown to play a role in regulating VEGF production (Nakamura K 2006), it would be of interest to investigate the existence of autocrine and paracrine mechanisms that may control physiological responses to neuronal injury in retinal disease.

Conclusion

This study adds to the growing recognition of the importance of Müller cells in the maintenance and support of other retinal cell types by producing or assisting in trafficking of neurotrophins. NT expression may be enhanced, as shown in PVR specimens or diminished as demonstrated in melanoma affected eyes and laser induced injury. A substantial increase in expression of one NT may lead to reduction of availability of its receptor and Müller cells may be involved in internalization and transport of NT in the retina.

Functional changes in RPE cells, demonstrated by changes in CD68 expression and down regulation of the NT4 in the ONL may be responsible for some of the changes seen in the retinal function after laser induced injury. Alterations in the expression of other neurotrophins and their receptors may also play a role in the response of the retina to various injuries. Further studies are needed to investigate the role of neurotrophins in the pathobiology of wound repair in the retina and possible role of NT4 in PVR.

Cheng L *et al* (2002) have shown that availability of TrK receptors is very important if exogenous (or endogenous) NTs are to have their supportive effect. There is growing interest in intraocular delivery of various growth factors and NTs to promote neuronal survival. Findings of this study show increased NT4 staining and reduced TrkB detection in PVR specimens. These findings support previous reports and indicate that delivery of NTs on their own may not be an adequate strategy for neuronal support.

Appendix

BDNF gene sequence (Jones KR 1990)

1 ggtgaaagaa agccctaacc agttttctgt cttgtttctg ctttctccct acagtccac
61 caggtgagaa gagtgatgac catccttttc cttactatgg ttatttcata ctttggttgc
121 atgaaggctg ccccatgaa agaagcaaac atccgaggac aaggtggctt ggcctacca
181 ggtgtgcgga cccatgggac tctggagagc gtgaatgggc ccaaggcagg ttcaagaggc
241 ttgacatcat tggctgacac tticgaacac gtgatagaag agctgttgga tgaggaccag
301 aaagttcggc ccaatgaaga **aaacaataag gacgcagact** tgtacacgtc cagggtgatg
361 ctcatagtc aagtgccttt ggagcctcct cttctcttc tgctggagga atacaaaaat
421 tacctagatg ctgcaaacat gtccatgagg gtccggcgcc actctgacc tgcccgcga
481 **ggggagctga** gcggtgtga cagtattagt gaggggtaa cgg**cggcaga caaaaagact**
541 **gcag**tggaca tgcgggcgg gacggtcaca gtcttgaaa aggtccctgt atcaaaaggc
601 caactgaagc aatacttcta cgagaccaag tgcaatccca tgggttacac aaaagaaggc
661 tgcaggggca tagacaaaag gcattggaac tccagtgcc gaactacca gtcgtacgtg
721 cgggccctta ccatggatag caaaaagaga attggctggc gattcataag gatagacact
781 tcttgtgtat gtacattgac cattaaaagg ggaagatagt ggatttatgt tgtatagatt
841 agattatatt gagacaaaaa ttatctattt gtatatatac ataacagggt aaattattca
901 gttaagaaaa aaataatt

Primer binding sites are highlighted in red.

NT4 gene sequence (Ip NY 1992)

1 cttgtcacc aggtggcagg ggagtgggtgc actctctgct cactgcaacc tcggcctcct
61 ggggtcagat gatttccta cctcagccta ctgagtagct gggattacag gcgtgcagca
121 ctatgcccg ttaatttgg tatttttgg agagatgagg ttccaccatg ttgaccagct
181 gctctggaac tctgaccte aagtcacca cctgcctcag cctccagag tgcaggatt
241 agaggtgtgg ggcacagtgc ctggcctgta gtagttgaat atttattatt aatctacaag
301 ttgcgcatta cgcaagccct agatatagg tccccaaac ttctagaaca agggcttccc
361 cacaatcctg gcaggcaagc ctcccctggg gtcccaact ttttccca ctgaagttt
421 taccctctc tctaaccga gctccctct tctgtctcc aggtgctcc agagatgctc
481 cctctccct catgctcct cccatcctc ctctttcc tctcccag tgtgccaatt
541 gaggcccaac cccaccctc aacattgcc cttttctgg cccctgagt ggaccttctc
601 tcccccgag tagtctgtc taggggtgcc cctgctggg cccctctgt ctctctgtg
661 gaggtgagg ctttcggga gtcagcagg gcccggcca accgcagcc gcgtggggtg
721 **agcgaaactg caccagcag** tcgtcgggt gagctggctg tgtcgatgc agtcagtggc
781 tgggtgacag accgccggac cgctgtggac ttgcgtggg gcgaggtgga ggtgttggc
841 gaggtgctg cagctggcg cagtcctc cgccagtact tcttgaaac ccgtgcaag
901 **gtgataacg ctgaggaagg** tggcccggg gcaggtggag ggggctgcc gggagtggac
961 aggaggcact ggggtatga gtgcaaggc aagcagct atgtcgggc attgaccgt
1021 gatgccagg gccgtgtgg ctggcgatg attcgaattg aactgcctg cgtctgcaca
1081 ctctcagcc ggactggcg ggctgagac ccatgccag gaaaataca gagctggatg
1141 ctgagagacc tcagggatg ccagctgat ctaaggacc cagttggga actcatcaa
1201 taatcaciaa atcacaattc tctgatttg agtcaatct ctgcaggat ggtgaaacca
1261 catgggggtt tggaggtga ataggagttc tctggagca actgagggt aataatgatg
1321 atgatataa aataatagc actatttact gagtgttac tgtttctat ccctaataca
1381 taactctca gatcaactct catg

Primer binding sites are highlighted in red.

t-TrkB gene sequence (Shelton DL 1995)

1 ggaaggttta aagaagaagc cgcaaagcgc agggaaggcc tcccggcacg ggtgggggaa
61 agcggccggt gcagcgcggg gacaggcact cgggctggca ctggctgcta gggatgtcgt
121 cctggataag gtggcatgga cccgccatgg cgcggctctg gggcttctgc tggtggttg
181 tgggcttctg gagggccgct ttcgcctgtc ccacgtcctg caaatgcagt gcctctcgga
241 tctgggtcag cgacccttct cctggcatcg tggcatttcc gagattggag cctaacagt
301 tagatcctga gaacatcacc gaaatttca tcgcaaacca gaaaaggta gaaatcatca
361 acgaagatga tgtgaagct tatgtgggac tgagaaatct gacaattgtg gattctggat
421 taaaatttgt ggctcataaa gcatttctga aaaacagcaa cctgcagcac atcaatttta
481 cccgaaacaa actgacgagt ttgtctagga aacatttccg tcacctgac ttgtctgaac
541 tgatcctggg gggcaatcca ttacatgct cctgtgacat tatgtggatc aagactctcc
601 aagaggctaa atccagtcca gacactcagg attgtactg cctgaatgaa agcagcaaga
661 atattcccct ggcaaacctg cagataccca attgtggtt gccatctgca aatctggccg
721 cacctaacct cactgtggag gaaggaaagt ctatcacatt atcctgtagt gtggcagggt
781 atccggttcc taatatgtat tgggatgttg gtaacctggg ttccaaacat atgaatgaaa
841 caagccacac acagggtcc ttaaggataa ctaacatttc atccgatgac agtgggaagc
901 agatctcttg tgtggcggaa aatctttagt gagaagatca agattctgtc aacctcactg
961 tgcattttgc accaactatc acatttctcg aatctccaac ctacagaccac cactggtgca
1021 ttccattcac tgtgaaaggc aacccaaaac cagcgttca gtggttctat aacgggggcaa
1081 tattgaatga gtccaaatac atctgtacta aaatacatgt taccaatcac acggagtacc
1141 acggtgcct ccagctggat aatcccactc acatgaacaa tggggactac actctaatag
1201 ccaagaatga gtatgggaag gatgagaaac agatttctgc tacttcatg ggctggcctg
1261 gaattgacga tgggtcaaac ccaaattatc ctgatgtaat ttatgaagat tatggaactg
1321 cagcgaatga catcggggac accacgaaca gaagtaatga aatcccttcc acagacgtca
1381 ctgataaaac cggtcgggaa catctctcgg tctatgctgt ggtggtgatt gcgtctgtgg
1441 tgggattttg ccttttggtg atgctgttcc tgettaagtt ggcaagacac tccaagtttg
1501 gcatgaaag tttgtttg ttcca laaga tcccactgga tgggtagctg aaataaagga
1561 aaagacagag aaaggggctg tgggtgctgt tggttgatgc tgccatgtaa gctggactcc
1621 tgggactgct gttggcttat cccgggaagt gctgcttacc tggggtttcc tggtagatgt
1681 gggcggtgtt tggaggctgt actatatgaa gcctgcatat actgtgagct gtgattgggg
1741 aacaccaatg cagaggtaac tctcaggcag ctaagcagca cctcaagaaa acatgttaaa
1801 ttaatgcttc tctcttaca gtagttcaaa tacaaaactg aaatgaaatc ccattggatt
1861 gtacttctct

Red; primers produce 166bp product (Labouyrie E 1999)

Yellow; primers produce 430bp product (Wordinger R 2000)

Green; sequence identical to full length receptor up to and including nucleotide 1510

TrkB gene sequence (Shelton DL 1995)

1 ggaagggtta aagaagaagc cgcaaagcgc agggaaggcc tcccggcacg ggtgggggaa
61 agcggccggt gcagcgcggg gacaggcact cgggctggca ctggctgcta gggatgtcgt
121 cctggataag gtggcatgga cccgccatgg cgcggctctg gggcttctgc tggctgggtg
181 tgggcttctg gagggccgct ttcgctgtc ccacgtctg caaatgcagt gcctctcgga
241 tctggtgcag cgacccttct cctggcatcg tggcatttcc gagattggag cctaacagtg
301 tagatcctga gaacatcacc gaaattttca tcgcaaacca gaaaaggta gaaatcatca
361 acgaagatga tgttgaagct tatgtgggac tgagaaatct gacaattgtg gattctggat
421 taaaatttgt ggctcataaa gcatttctga aaaacagcaa cctgcagcac atcaatttta
481 cccgaaacaa actgacgagt ttgtctagga aacatttccg tcaccttgac ttgtctgaac
541 tgatcctggt gggcaatcca ttacatgct cctgtgacat tatgtggatc aagactctcc
601 aagaggctaa atccagtcca gacactcagg atttgactg cctgaatgaa agcagcaaga
661 atattcccct ggcaaacctg cagataccca attgtgggtt gccatctgca aatctggccg
721 cacctaacct cactgtggag gaaggaaagt ctatcacatt atcctgtagt gtggcagggtg
781 atccggttcc taatatgtat tgggatgtt gtaacctggt ttccaaacat atgaatgaaa
841 caagccacac acagggctcc ttaaggataa ctaacatttc atccgatgac agtgggaagc
901 agatctcttg tgtggcggaa aatctttagt gagaagatca agattctgtc aacctcactg
961 tgcattttgc accaactatc acatttctcg aatctccaac ctcagaccac cactggtgca
1021 ttccattcac tgtgaaaggc aaccctaaaac cagcgttcca gtggttctat aacggggcaa
1081 tattgaatga gtccaaatc **atctgtacta aaatacat**gt taccaatcac acggagtacc
1141 acggtctgct ccagctggat aatcccactc acatgaacaa tggggactac actctaatag
1201 ccaagaatga gtatgggaag gatgagaaac agatttctgc tcacttcatg ggctggcctg
1261 gaattgacga tggtgcaaac ccaattatc ctgatgtaat ttatgaagat tatggaactg
1321 **cagcgaatga catcggggac accac**gaaca gaagtaatga aatccctcc acagacgtca
1381 ctgataaaac cgtcggggaa catctctcgg tctatgctgt ggtggtgatt gcgtctgtgg
1441 tgggattttg ccttttggta atgctgttct tgccttaagt ggcaagacac tccaagtttg
1501 gcatgaaagg cccagcctcc gttatcagca atgatgatga ctctgccagc ccactccatc
1561 acatctccaa tgggagtaac actccatctt ctccggaagg tggcccagat gctgtcatta
1621 ttggaatgac caagatccct gtcattgaaa atccccagta ctttggcatc accaacagtc
1681 agctcaagcc agacacattt gttcagcaca tcaagcgaca taacattgtt ctgaaaaggg
1741 agctaggcga aggagccttt ggaaaagtgt tctagctga atgtataac ctctgtcctg
1801 agcaggacaa gatcttgggt gcagtgaaga cctgaagga tgccagtgc aatgcacgca
1861 aggacttcca cgtgaggcc gagctcctga ccaacctcca gcatgagcac atcgtcaagt
1921 tctatggcgt ctgcgtggag ggcgacccc tcatcatggt ctttgagtac atgaagcatg
1981 gggacctcaa caagtctctc agggcacacg gccctgatgc cgtgctgatg gctg**agggca**
2041 **acccgcccac gga**actgacg cagtcgaga tgctgcatat agcccagcag atcgccgcgg
2101 gcatggtcta cctggcgtcc cagcacttgc tgcaccgca ttggccacc aggaactgcc
2161 tggtcgggga gaacttgctg gtgaaaatcg gggactttgg gatgtcccgg gacgtgtaca
2221 gactgacta ctacagggtc ggtggccaca caatgctgcc cattgctgg atgcctccag
2281 agagcatcat gtacaggaac ttacgacgg aaagcgacgt ctggagcctg ggggtcgtgt
2341 tgtgggagat ttacacctat ggcaaacagc cctggtacca gctgtcaaac aatgaggtga
2401 tagagtgtat cactcagggc cgagtcctgc agcgacccc cactgcccc caggaggtgt
2461 atgagctgat gctgggggtg tggcagcgag agccccacat gaggaagaac atcaagggca
2521 tccataacct ccttcagaac ttggccaagg catctccggt ctacctggac attctaggct
2581 agggc**ccttt tccccagacc gatc**cttccc aacgtactcc tcagacgggc tgagaggatg
2641 aacatctttt aactgccgct ggaggccacc aagctgctct ccttactct gacagtatta
2701 acatcaaaga ctccgagaag ctctcgaggg aagcagtgtg tacttttca tccatagaca
2761 cagtattgac ttctttttg cattatctct ttctctttt ccatctccct tggttgtcc

2821 tttttctttt tttaaatttt ctttttcttc tttttttcg tcttccctgc ttcacgattc
2881 ttaccctttc ttttgaatca atctggcttc tgcattacta ttaactctgc atagacaaag
2941 gccttaacaa acgtaatttg ttatatcagc agacactcca gtttgcccac cacaactaac
3001 aatgccttgt tgtattcctg cctttgatgt ggatgaaaaa aagggaaaac aaatatttca
3061 cttaaacttt gtcacttctg ctgtacagat atcgagagtt tctatggatt cacttctatt
3121 tatttattat tattactggt cttattgttt ttggatggct taagcctgtg tataaaaaaa
3181 aaaaaaaatc taga

Extra cellular domain primers position 1098-1117 (highlighted in red)

Kinetic domain primers position 2035-2054 (highlighted in yellow)

GAPDH gene sequence (Strausberg R 2002)

```
1 cagtcagccg catcttcttt tgcgtcgcca gccgagccac atcgctgaga caccatgggg
61 aaggtgaagg tcggagtcaa cggatttggg cgtattgggc gcctggtcac cagggtgct
121 tttactctg gtaaagtga tattgttgcc atcaatgacc cttcattga cctcaactac
181 atggtttaca tgtccaata tgattccacc catggcaaat tccatggcac cgtcaaggct
241 gagaacggga agcttgcac caatggaaat cccatcacca tctccagga gcgagatccc
301 tccaaaatca agtggggcga tgctggcgt gagtacgtc tggagtccac tggcgtcttc
361 accaccatgg agaaggctgg ggctcattg caggggggag caaaagggt catcatctct
421 gccccctctg ctgatcccc catgttcgc atgggtgtga accatgagaa gtagacaac
481 agcctcaaga tcacagcaa tgcctctgc accaccaact gcttagcacc cctggccaag
541 gtcacccatg acaacttgg tctcgtggaa ggactcatga ccacagtcca tgccatcat
601 gccaccaga agactgtgga tggcccccc gggaaactgt ggcgtgatgg ccgcgggggt
661 ctccagaaca tcaccctgc ctctactgc gctgccaagg ctgtgggcaa ggtcatccct
721 gagctgaacg ggaagctcac tggcatggcc ttccgtgtcc cactgcca cgtgtcagt
781 gtggacctga cctgccgtct agaaaaacct gccaaatatg atgacatcaa gaaggtggtg
841 aagcaggcgt cggagggccc cctcaagggc atcctgggct acactgagca ccaggtggtc
901 tctctgact tcaacagcga caccactcc tccaccttg acgtggggc tggcattgcc
961 ctcaacgacc actttgtcaa gctcattcc tggtatgaca acgaatttg ctacagcaac
1021 aggtgtggtg acctcatggc ccacatggcc tccaaggagt aagaccctg gaccaccagc
1081 cccagcaaga gcacaagagg aagagagaga cctcactgc tggggagtc ctgccacact
1141 cagtccccc ccacactgaa tctccctcc tcacagtgc catgtagacc ccttgaagag
1201 gggaggggccc tagggagccg cacctgtca tgtaccatca ataaagtacc ctgtgtcaca
1261 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa a
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Primer binding sites are highlighted in red.

Supporting publications

Peer review publication

Ghazi-Nouri SM, Assi A, Limb GA, Scott RA, von Bussmann K, Humphrey I et al. Laser photocoagulation alters the pattern of staining for neurotrophin-4, GFAP, and CD68 in human retina. *Br.J.Ophthalmol.* 2003;87:488-92.

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